Dietary strategies to reduce methane emissions from ruminants

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Abstract

Ruminant products form an important part of the human diet. The demand for ruminant products is expected to increase due to the increase in the size of the human population and its increasing wealth. The production of ruminant meat and milk is associated with a relatively large environmental impact when compared to other animal products. This is, for a large part, caused by the fact that ruminants produce enteric methane, a greenhouse gas, during the digestion of their feed. Many dietary strategies have been proposed to lower methane production in ruminants, although most of these have only been tested in vitro. In this thesis, a number of dietary strategies, that had been proven effective in vitro, were evaluated for their in vivo efficacy in methane reduction. A mixture of lauric acid, myristic acid, linseed oil and calcium fumarate lowered methane production by 10% in lactating dairy cows. However, fat and protein corrected milk production was negatively affected by feeding this mixture. Despite the methane reduction, energy balance was unaltered in this study. Diallyldisulfide, yucca powder, calcium fumarate, an extruded linseed product and a mixture of capric and caprylic acid did not affect methane production in lactating dairy cows, although their efficacy had been demonstrated in vitro. The addition of nitrate and sulfate to sheep diets lowered in vivo methane emissions (-32% and -16%, respectively), presumably by acting as a hydrogen sink in the rumen. No negative side-effects of feeding nitrate or sulfate were observed in this study. The use of nitrate in methane mitigation was further evaluated in a long-term study with dairy cows. Dietary nitrate persistently lowered methane production by 16% in dairy cows over the 89-d experimental period. Despite this reduction in methane production, milk production or energy retention were not improved. Methemoglobin levels in blood were slightly elevated, when nitrate was fed to dairy cows. Further analysis of the efficacy of nitrate in methane mitigation demonstrated that the efficacy of nitrate in methane mitigation decreased with increasing dose of nitrate (expressed in g nitrate/$kg^{0.75}$ per day). The conversion of metabolizable energy gained from a lowering of methane production may be less efficient than is commonly assumed. This could originate from a shift from methane to hydrogen emissions, when methane is specifically inhibited, or from erroneous assumptions made in the calculation of heat production during indirect respiration calorimetry. Dietary fat addition may be an effective strategy to lower methane production from ruminants, although the fatty acid profile of the added fat does not appear to have additional effects on methane production from ruminants. When assessing the environmental impact of ruminant products, it is generally overlooked that ruminants are capable of transforming feed not accessible to humans into human food.

Voorwoord

Zo hier zit ik dan, het einde van een tijdperk! Ik had zelf niet gedacht ooit te gaan promoveren, maar de combinatie van het onderwerp en de mogelijkheid het onderzoek namens een commercieel bedrijf te kunnen doen, hebben me ruim vier jaar geleden toch doen besluiten eraan te beginnen. Het is een mooie reis geweest, waarop ik vele mensen heb leren kennen, zonder wie dit proefschrift niet geworden was wat het nu is en zonder wie het uitvoeren van het onderzoek ook een stuk minder leuk was geweest.

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Opgedragen aan mijn vader.

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CHAPTER 1

General Introduction

The Ruminant and Its Role in Human Nutrition

Ruminants can be distinguished from other mammalian species by their highly specialized, compartmentalized stomach system. The largest of the four stomachs is called the rumen, where a symbiotic population of microflora and –fauna secretes enzymes that allow breakdown of complex structural cell wall components of plants and other ingested components by the host ruminant. Subsequently, the breakdown products, mainly sugars and amino acids, are fermented by microbes. This fermentation yields energy for the microbes and the resulting fermentation end-products, volatile fatty acids (VFA), are absorbed by the ruminant and used as its main energy source. The microbial biomass forms a valuable source of protein to the ruminant as it flows out of the rumen to be digested in the small intestine. During the fermentation process, methane is formed as a by-product and is exhaled and eructed by the host animal. The ability to break down and utilize fibrous plant material allows the ruminant to survive on plant substrates that are mostly inaccessible to mammalian enzymes and has allowed the ruminant to occupy a specific niche in the animal kingdom (Van Soest, 1994).

The first ruminants evolved around 50 million years ago, and their domestication started around 10,000 B.C. with the husbandry of goats for meat production (Zeder and Hesse, 2000). Their purpose was initially for meat production, but later, ruminants were also used for milk production, draught power, transportation, currency and in religious rituals (Clutton-Brock, 1999). The global population of domesticated ruminants is currently estimated at 3.6 billion, nearly 50 times as large as the population of wild ruminants (Hackmann and Spain, 2010).

Ruminants nowadays play an important role in human nutrition. They are the predominant source of milk for human nutrition (487 million metric tons in 2001; Steinfeld et al., 2006) and supplied approximately 36% of the global meat for human consumption in 1990 (Rosegrant et al., 1995). Although per capita milk and meat consumption have mostly stabilized in the developed countries, meat and milk consumption are likely to increase in the emerging economies as the size of their population increases and increasing wealth and urbanization increases the proportion of animal products in their diets (Steinfeld et al., 2006). Global milk consumption is anticipated to increase to 736 million metric tons in 2030 and meat consumption to 373 million metric tons per annum (Steinfeld et al., 2006). Assuming that ruminant meat will continue to represent the same share of this consumption, the demand for ruminant meat and milk will increase by more than 50% over the next 2 decades.

Methanogenesis in Anaerobic Environments

In anaerobic environments, organic material is decomposed by bacteria through the process of fermentation, where organic material is broken down to, among others, VFA and carbon dioxide. Hydrogen, released during the production of VFA, accumulates in the fermentation system. In an aerobic environment, oxygen would be the terminal electron acceptor and oxygen would be reduced to H_2O, using the excess hydrogen in the process. The lack of oxygen in anaerobic systems necessitates the use of other terminal electron acceptors to remove hydrogen from the fermentation system. A number

of compounds can be used for this purpose (ferric iron, sulfate, nitrate, manganese and carbon dioxide). However, these compounds, except for carbon dioxide, are only present in low concentrations in most fermentation systems. The compounds that are normally present in low concentrations (ferric iron, sulfate, nitrate and manganese) are rapidly reduced and exhausted and carbon dioxide functions as the main terminal electron acceptor for the remaining excess hydrogen. Carbon dioxide is reduced to methane in the fermentation system (equation 1), and the methane in gaseous form subsequently dissipates from the system.

$$CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O \qquad \text{(equation 1)}$$

Methane production occurs during the fermentation of organic matter in a wide variety of environments, including the intestinal tract of animals, fresh water sediments, wetlands, swamps, landfills, termite guts, rice fields and animal manure storage (Deppenmeier, 2002). In all these different environments, the production of methane serves an important function; it removes hydrogen from the site of fermentation by reduction of carbon dioxide and maintains a low redox potential at the site of fermentation. Without the removal of hydrogen, re-oxidation of reduced cofactors (NADH, NADPH and FADH) would be inhibited by the accumulated hydrogen and the production of VFA would be inhibited (Wolin, 1975). Methane is produced by a very specific group of micro-organisms, called the methanogenic Archaea (Chaban et al., 2006).

Enteric Methanogenesis in Ruminants

Fermentation and VFA production by the microbes in the intestinal tract of animals is accompanied by the production of methane. Many omnivorous and herbivorous mammals partly depend on microbial fermentation to digest their feed. Consequently, many mammals, including the domesticated species for meat and milk production, produce methane during feed digestion. However, the extent of dependency on microbial fermentation for feed digestion varies widely among species and, consequently, the amount of methane produced per animal also varies widely. Ruminants rely on microbial fermentation to a larger extent than other species and methane emissions from ruminant species expressed per kilogram of body weight are relatively high for this reason (*Table 1.1*).

Table 1.1

Estimated annual enteric methane emissions from the main domesticated livestock species (Sauvant, 1993).

	Methane emission ($kg\ CH_4\ animal^{-1}\ year^{-1}$)	**Assumed average** bodyweight (kg)	**Methane emission** ($g\ kg\ BW^{-1}\ year^{-1}$)
Ruminants			
Dairy cows	90	600	150
Beef cattle	65	400	163
Sheep	8	50	160
Goats	8	50	160
Non-ruminants			
Swine	1	80	13
Poultry	<0.1	2	-
Horses	18	600	30

In the ruminant, bacterial fermentation occurs both in the rumen as well as in the hindgut. Enteric fermentation in the large intestine of ruminants has been estimated to account for 13% of the total enteric methane emissions and the majority of the methane thus originates from the rumen (Murray et al., 1976). The quantity of methane produced per unit of fermented feed is proportional to the pattern of the VFA produced. The three main VFA produced during rumen fermentation are acetate, propionate and butyrate. During the production of acetate (equation 2) and butyrate (equation 4), hydrogen is produced, but the production of propionate (equation 3) results in the net uptake of hydrogen. A higher proportion of propionate in the VFA-profile therefore results in reduced methane production (Ellis et al., 2008) and this property can be utilized in the manipulation of methane production. However, the production of acetate and butyrate always exceeds propionogenesis, resulting in a net surplus of hydrogen in the rumen.

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2C_2H_3O_2^- + 2CO_2 + 4H_2 + 2H^+ \quad \text{(equation 2)}$$

$$C_6H_{12}O_6 + 2H_2 \rightarrow 2C_3H_5O_2^- + 2H^+ + 2H_2O \quad \text{(equation 3)}$$

$$C_6H_{12}O_6 \rightarrow C_4H_7O_2^- + H^+ + 2H_2 + 2CO_2 \quad \text{(equation 4)}$$

Typically, a lactating dairy cow loses 6.5% of the gross energy she ingests through methane emissions (IPCC, 2006), but considerable variation around this number exists, partly depending on diet composition (Johnson and Johnson, 1995). Much research has been devoted to reducing methane emissions and to enhance the energy capture from the ruminant diet (e.g. Blaxter and Czerkawski, 1966, Czerkawski et al., 1966).

Ruminants and Global Warming

Over the past decades concern has arisen over the accumulation of gases in the atmosphere that are capable of trapping heat (Figure 1.1), leading to increased average global temperatures. It is highly likely that these so-called greenhouse gases have increased in

concentration in the atmosphere due to the increased size of the human population and its concomitant activities (Forster et al., 2007).

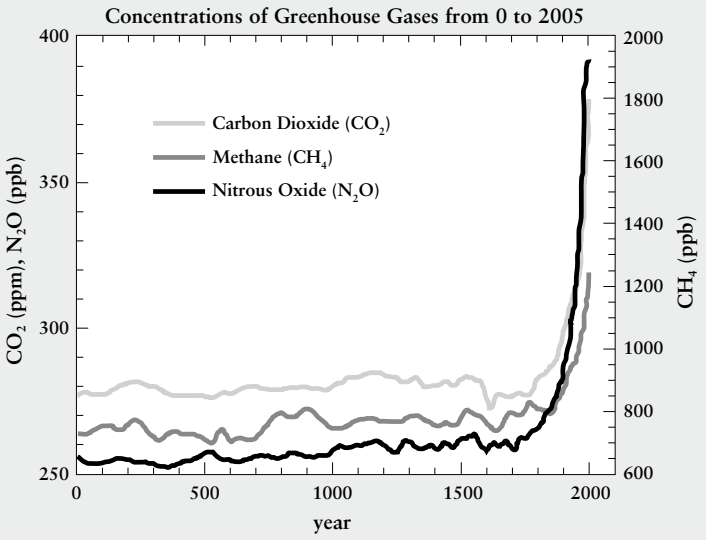

Figure 1.1

Atmospheric concentrations of the three main greenhouse gases carbon dioxide, methane and nitrous oxide in the period 0-2005 (Forster et al., 2007).

Quantitatively, the most important greenhouse gas is carbon dioxide and about 77% of global warming is attributed to the increased atmospheric concentration of this gas (Forster et al., 2007). The production of carbon dioxide mainly results from the burning of fossil fuels, which has increased dramatically since the onset of the industrial revolution. However, the atmospheric concentrations of most of the other greenhouse gases (methane, nitrous oxide, chlorofluorocarbons, hydrochlorofluorocarbons, hydrofluorocarbons, perfluorocarbons and sulphur hexafluoride) have also increased over the past decades (Forster et al., 2007).

Methane is the second most important gas involved in global warming and accounts for 14% of the human-induced production of greenhouse gases (Forster et al., 2007). To be able to compare greenhouse gases, their effect on global warming is usually expressed relative to CO_2. Methane has global warming potential that is 23-25 times larger than CO_2 and therefore contains 23-25 CO_2-equivalents (Forster et al., 2007). Methane emissions are divided into two types; natural emissions, which have always occurred and anthropogenic emissions, which have been induced by the human population. The emission of methane from domesticated ruminants is a consequence of the demand

for animal products for human consumption and is thus part of the anthropogenic greenhouse gas emissions.

Agricultural activities are responsible for 37% of anthropogenic methane emissions, with enteric methane emissions from ruminants representing the largest share (86 million tons or 23% of anthropogenic methane emissions; (Steinfeld et al., 2006)). The global dairy sector has recently been estimated to contribute 4.0% of the globally produced greenhouse gases with more than 50% coming from methane (FAO, 2010).

The relatively large contribution of enteric fermentation to the global production of greenhouse gases and the projected increase in demand for ruminant products have led to the initiation of many programs to assess strategies to reduce methane emissions from ruminants. Taking into account the increase in future demand for animal products, greenhouse gas emissions per unit of animal product will have to be more than halved in order to just maintain the current impact of animal husbandry on global warming (Steinfeld et al., 2006).

Strategies to Reduce Enteric Methane Emissions

When expressed per kg of ruminant product (milk and meat), methane emissions vary widely between different parts of the world. This is largely a consequence of the level of productivity in different geographical regions in the world (FAO, 2010). In the developed world, genetic progress and improved feeding management have considerably improved animal productivity over the past decades. Although methane emissions per animal have generally increased, per unit of consumable product (milk and meat), methane emission has decreased significantly.

For example, in the United States, a dairy cow produced 13.5 kg CO_2-equivalents/d in 1944. In 2007, greenhouse gas production per cow had more than doubled to 27.8 kg CO_2-eq./d. However, milk production increased from 2070 kg/yr to 9152 kg/yr and the carbon footprint of milk was reduced from 3.66 kg CO_2 eq./kg milk in 1944 to 1.35 kg CO_2-eq./kg milk in 2007 (Capper et al., 2009). The figures for direct emissions by dairy cows were based on direct emissions of greenhouse gases from the dairy cow, whereas the figures for milk also include greenhouse gas emissions from other animals (dry cows and young stock), cropping, fertilizer use and manure. Although these figures were estimated using a fairly simplistic model, they demonstrate the potential of increasing productivity to reduce greenhouse gas emissions.

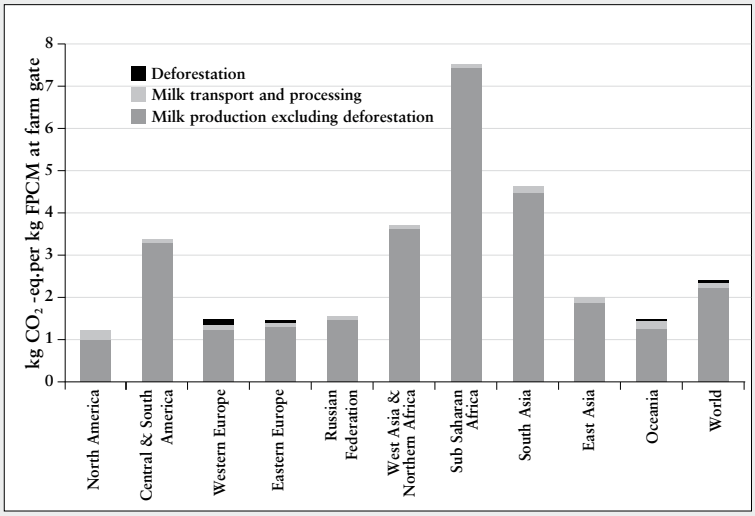

Figure 1.2

Greenhouse gas emissions of milk produced in different regions in the world (FAO, 2010).

By adopting better management practices in countries that currently have a low level of milk production, it is likely that the carbon footprint per unit of milk can be substantially reduced (Figure 1.2). As a consequence, the increase in production of greenhouse gases associated with ruminant production will probably increase at a slower pace than the demand for ruminant products. However, additional measures will still be necessary to avoid large increases in greenhouse gas emissions from ruminant husbandry systems and large research programs have been initiated globally to explore dietary strategies to directly lower enteric methanogenesis.

Research to specifically reduce methane emission from ruminants was first initiated in the early 60's to reduce the dietary energy losses that are associated with methane production. Much research has subsequently been published in the 60's and 70's to investigate the potential to reduce methane emissions and reduce the energetic losses from methane production. After a period of less research activity on this topic in the 80's, methane research resurged in the 90's and it has been an active area of research ever since. The main focus however, has shifted in recent years from the reduction of methane emissions to benefit the animals' energy utilization to the reduction of methane as a greenhouse gas.

Dietary strategies to reduce enteric methane emissions mainly revolve around one of the following principles (Joblin, 1999, Martin et al., 2010):

- Direct inhibition of methanogenesis
- Lowering of the production of hydrogen during fermentation
- Providing alternative pathways for use of hydrogen in the rumen

The potential of dietary strategies to reduce methane emission by ruminants has been extensively reviewed (Tamminga et al., 2007, Beauchemin et al., 2008, Martin et al., 2010, Eckard et al., 2011). No attempt will be made to duplicate these efforts in this thesis, but the main strategies resulting from these reviews are briefly discussed below.

Many plant extracts (e.g. tannins, saponins, essential oils) have been screened in vitro for their potential to directly inhibit methanogenesis e.g. (Calsamiglia et al., 2007, García-González et al., 2008). The results of these screenings are very promising, but in vivo verification of their efficacy remains necessary. The in vivo effectiveness of two plant components able to lower methane production in vitro, Yucca powder, and diallyldisulfide, is investigated in Chapter 3 of this thesis.

Ionophores are widely used in ruminant nutrition, although they are not permitted for use in the European Union. Monensin, a commonly used ionophore in ruminant nutrition shifts the VFA-pattern in the rumen towards propionate, thereby providing an alternative hydrogen sink. The long-term persistency of Monensin in methane mitigation is not clear, with studies demonstrating a persistent effect (Odongo et al., 2007), while another study showed the effect to be transient (Guan et al., 2006).

The use of organic acids has been proposed as a means to provide an alternative hydrogen sink to methanogenesis. Malate and fumarate can be converted into propionate in the rumen, consuming hydrogen in the process. A number of in vivo studies with these compounds have been performed, but the effects on methane production have been conflicting (Bayaru et al., 2001, Kolver and Aspin, 2006, Wallace et al., 2006, Wood et al., 2009). In this thesis, fumarate is evaluated for its effectiveness in reducing methane emissions from ruminants (Chapters 2 and 3). The use of nitrate and sulfate as alternative hydrogen sinks has largely been ignored, because intermediary products that are formed during the reduction of these compounds can be harmful to animals (Bruning-Fann and Kaneene, 1993). In this thesis, the use of these products as alternative hydrogen sinks to methanogenesis is explored (Chapters 4 and 5).

Dietary fat is not fermented in the rumen and, consequently, less hydrogen per unit of feed is produced when higher fat levels are included in the diets for ruminants. Increasing the dietary fat content has therefore been proposed as a promising strategy to reduce methane emissions from ruminants (Eugene et al., 2008, Martin et al., 2010). Moreover, individual fatty acids have been considered to have specific anti-methanogenic properties, and methane production could be further reduced by using these specific fatty acids (Czerkawski et al., 1966, Ajisaka et al., 2002, Machmüller, 2006). This concept is explored in the experiment described in Chapter 3, in which different fat sources, with different fatty acid profiles, are evaluated for their anti-methanogenic properties at equal dietary fat levels.

Measurement of Methane Production from Ruminants

The measurement of methane emissions from individual animals is complex, because of the gaseous nature of the methane emitted. A number of techniques have been developed to measure methane emissions from animals. These include methane estimations from the VFA production (Hegarty and Nolan, 2007), isotopic (Hegarty et al., 2007) and non-isotopic tracer techniques (Johnson et al., 1994), ventilated hood techniques (Odongo et al., 2007) and measurements in confined respiration chambers (Frankenfield, 2010). Besides these techniques, methane emissions can also be determined employing meteorological techniques, like the tunnel technique (Murray et al., 2007) or area up-downwind techniques (Judd et al., 1999).

The 2 most commonly employed techniques are the respiration chamber technique and SF_6-tracer technique. The respiration chamber technique (Verstegen et al., 1987) entails confining 1 or more animals in airtight chambers, from which the in and out flowing air is monitored for gases (oxygen, carbon dioxide, methane and hydrogen). The benefit of employing this technique is the high accuracy of the measurements. Also, manure and urine can be collected from these chambers, allowing for determination of feed digestibility and determination of full energy balance. A disadvantage of this technique is that animals are confined in chambers, which may alter their behavior and this could potentially also affect gaseous emissions. This method does not allow for measurement of methane emissions from grazing animals. Another disadvantage of this technique is the high cost of operation.

The SF_6 tracer-technique was specifically designed to measure methane from ruminants (Johnson et al., 2007). A permeation tube containing the tracer gas sulfur hexafluoride (SF_6) is inserted into the rumen of the animal. The release rate of SF_6 from the permeation tube is carefully determined before insertion of the tube into the animal. Subsequently, air is sampled from the nostrils of the cow into a vacuum collection canister over a 24 h-period. In the collection canister, a representative sample of the expired and eructed breath of the animal is collected. The air in the canister is analyzed for SF_6 and methane concentrations. Because the absolute amount of SF_6 released from the animal is known, the volume of methane produced can be calculated from the methane and SF_6-concentration in the canister and the known quantity of SF_6 released from the permeation tube. This technique allows for measurements in grazing animals and methane production is unlikely to be affect by changes in the animals' behavior.

The SF_6-technique has been compared with the respiration chamber technique and measurements with both techniques showed good correlations in most (Johnson et al., 1994, McGinn et al, 2006), but not all studies (Lassey et al., 1997, Wright et al., 2004). The SF_6-technique is known to generally underestimate methane emissions relative to the chamber technique and to have a larger standard deviation (Johnson et al., 1994, McGinn et al, 2006). Variation in methane production within and between animals is more pronounced with the SF_6 than the chamber technique (Pinares-Patinho and Clark, 2008). For this reason, more animals will have to be included in a study using this technique rather than the chamber technique.

In this thesis, the production of methane was determined using the respiration chamber technique. This technique was chosen because of its higher accuracy and the possibility to quantify the effect of lowering of methane production on the overall energy balance of the animal.

Aims and Contents of this Thesis

Many dietary strategies to reduce methane emissions from ruminants have already been suggested from in vitro research, but these have not often been verified in vivo. In addition, rumen microbes have been demonstrated to be highly adaptive to changes in dietary strategies (Guan et al., 2006) and the persistency of a methane lowering strategy can be questionable. The in vitro studies generally last from a few days until a week, which allows the rumen fluid little time to adapt.

In this thesis, various strategies to reduce enteric methane emissions, that have previously been shown effective in vitro, are evaluated for their potential use in live animals in relatively short term experiments (approximately 3 weeks). This experiment duration was chosen as an optimum to allow time for microbial adaptation and still have relatively short term research. The most promising strategy that was effective in vivo was further evaluated for its persistency in reducing methane in a long term experiment (approximately 4 months). During the experiments feed intake and animal performance were evaluated as any negative impact on animal performance might negatively impact the success of the methane mitigation strategy. Also, complete energy balances were determined to evaluate the energetic benefit of methane reduction by dietary strategies.

The thesis aims to answer the following questions:

- Are selected dietary strategies to reduce methane emissions that have previously been shown to be effective in vitro, also effective in the in vivo situation?
- Will a reduction in enteric methane production as a consequence of feeding a dietary additive persist over time?
- Can the energy status of the animal be improved by reducing methanogenesis through methane reducing dietary strategies?

In Chapter 2, a mixture of dietary additives is evaluated for it methane reducing properties to demonstrate the potential of reducing methane emissions by addition of a combination of dietary additives. Chapter 3 deals with an analysis of the effect of individual feed additives on methane emissions. In Chapter 4, a first evaluation is made on the effectiveness of two alternative hydrogen sinks, nitrate and sulfate, in reducing methane emissions in sheep. The more effective of these two hydrogen sinks, nitrate, is further evaluated in dairy cows in Chapter 5. Apart from the immediate methane lowering potential, the persistency of the methane lowering effect of dietary nitrate is evaluated in Chapter 5.

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CHAPTER 2

Effects of a combination of feed additives on methane production, diet digestibility, and animal performance in lactating dairy cows

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ABSTRACT

Two experiments were conducted to assess the effects of a mixture of dietary additives on enteric methane production, rumen fermentation, diet digestibility, energy balance and animal performance in lactating dairy cows. Identical diets were fed in both experiments. The mixture of feed additives investigated contained lauric acid, myristic acid, linseed oil and calcium fumarate. These additives were included at 0.4%, 1.2%, 1.5% and 0.7% of dietary dry matter, respectively (treatment ADD). Experimental fat sources were exchanged for a rumen inert source of fat in the control diet (treatment CON) to maintain isolipidic rations. Cows (experiment 1; n = 20; experiment 2; n = 12) were fed restricted amounts of feed to avoid confounding effects of dry matter intake on methane production. In experiment 1, methane production and energy balance were studied using open circuit indirect calorimetry. In experiment 2, 10 rumen fistulated animals were used to measure rumen fermentation characteristics. In both experiments animal performance was monitored.

The inclusion of dietary additives reduced methane emissions (g/d) by 10%. Milk yield and milk fat content tended to be lower for ADD in experiment 1. In experiment 2, milk production was not affected by ADD, but milk fat content was lower. Fat- and protein-corrected milk was lower for ADD in both experiments. Milk urea nitrogen content was lowered by ADD in experiment 1 and tended to be lower in experiment 2. Apparent total tract digestibility of fat, but not that of starch or NDF, was higher for ADD. Energy retention did not differ between treatments. The decrease in methane production (g/d) was not evident when methane emission was expressed per kilogram of milk produced. Feeding ADD resulted in increases of C12:0 and C14:0 and the intermediates of linseed oil biohydrogenation in milk in both experiments.

In experiment 2, ADD-fed cows tended to have a decreased number of protozoa in rumen fluid when compared with that in control cows. Total volatile fatty acid concentrations were lower for ADD, whereas molar proportions of propionate increased at the expense of acetate and butyrate.

INTRODUCTION

Atmospheric methane concentrations have increased from a level of 715 ppb in the pre-industrial era to a level of 1,774 ppb in 2005 (Forster et al., 2007), probably due to an increase in the global human population and its concomitant production of greenhouse gases. Agricultural practice is responsible for a considerable part of anthropogenic methane production and enteric fermentation by ruminants is considered to be the single largest global source of anthropogenic methane emissions (86 million metric tons per annum; Steinfeld et al., 2006). Dietary composition has a large influence on the amount of methane produced by ruminants and dietary intervention is an interesting option to decrease methane emissions (Ellis et al., 2008). Methane losses can vary as much as 2 to 12% of the ingested gross energy by the animal (Johnson and Johnson, 1995).

Dietary fat addition has been shown to reduce methane production by ruminants in many studies (Jordan et al., 2006b, Machmüller, 2006, Martin et al., 2008). However, a recent meta-analysis (Eugene et al., 2008), showed that the decrease in methane emission caused by feeding fat to dairy cows was associated with a decrease in DMI. In addition, dietary fat addition will also decrease the proportion of fermentable organic matter (FOM) in the diet, which in itself would decrease methane emissions. Therefore, the potential of dietary strategies to decrease methane emissions should be evaluated at similar DMI and similar dietary fat content.

In the current experiments we evaluated the methane mitigating properties of a mixture of feed components (mainly lipids), while maintaining a similar dietary concentration of both FOM and lipid and a similar DMI. We hypothesized that part of the methane mitigating effects, reported previously for these additives were indirectly caused by differences in DMI or FOM concentration.

MATERIAL AND METHODS

Experimental Design

Experiment 1 was a randomized block design with 2 treatments involving 20 lactating Holstein-Friesian dairy cows with fat- and protein-corrected milk (FPCM) production of 32.8 ± 4.9 kg/d and 176 ± 76 DIM at the beginning of the experiment. Cows were paired (10 pairs of two cows) based on FPCM production, parity and DIM before the experiment. Within each pair, cows were randomly assigned to either the control treatment (CON) or the treatment with the additives (ADD). Treatment periods lasted 21 d. The experiment was carried out in 5 identical replicates. In each replicate, 4 cows (2 on each treatment) were housed in tie-stalls for a 14-d period to adapt to restriction in movement and the experimental diets. After the adaptation period, 2 cows, belonging to the same treatment group, were housed in respiration chambers for 7 d for measurement of methane production, apparent total tract digestibility of fat, starch and NDF, and energy and N balance. Two chambers were available and both treatments were tested in each of the replicates. The respiration calorimetry chambers were described in detail by Verstegen et al. (1987). Inside the chambers, temperature was maintained at 16°C and relative humidity at 70%. Ventilation rate was 34.8 m^3/h per cow and cows were

exposed to 16 h of light per day. Water was freely available during the entire experiment. Cows were milked and fed twice daily at 0600 and 1700 h. All data in this publication for experiment 1 pertain to the week the cows were in the respiration chambers. For methane production, apparent nutrient digestibility and energy balance data the experimental unit consists of the average data of two cows (n = 5 per treatment), whereas for production data (i.e., milk production, DMI) the experimental unit is the individual cow (n = 10 per treatment).

In experiment 2, the same dietary treatments (CON vs. ADD) were compared in a crossover design with 2 periods of 28 d, using 12 lactating Holstein-Friesian dairy cows with a milk production of 36.5 ± 8.0 kg/d and 216 ± 93 DIM at the beginning of the experiment. The allocation of cows to dietary treatments was similar to experiment 1. Cows were housed in tie-stalls. Animals were fed and milked twice daily (0600 and 1700 h). Water was freely available during the entire experiment.

Animals and Housing

The Animal Care and Use Committee of Wageningen University, the Netherlands approved the experimental protocol of experiment 1. Cows were housed at the experimental facilities of Wageningen University, the Netherlands. For the first 14 d of each period cows were housed in tie-stalls and they were subsequently moved to respiration chambers for the last 7 d of each period.

The Animal Care and Use Committee of the Animal Sciences Group, Lelystad, the Netherlands approved the experimental protocol of experiment 2. Twelve lactating Holstein-Friesian dairy cows were included in the experiment. Ten of these cows were rumen fistulated. Cows were housed in tie-stalls at the experimental facilities of the Provimi Research and Innovation Centre, Velddriel, the Netherlands.

Diets and Feeding

Diet formulation (*Table 2.1*) was identical in both experiments. Diets were formulated to be isolipidic and iso-energetic on a theoretical net-energy basis (Van Es, 1975). The fat source in CON (fractionated palmitic acid; Hyprofat, Provimi B.V., Rotterdam, The Netherlands) was assumed to be rumen inert because of its high melting point and thus assumed not to have an effect on rumen fermentation other than dilution of rumen FOM (Dohme et al., 2000, 2001). The fat sources in the ADD-treatment (lauric acid, myristic acid and linseed oil) have been shown to reduce methane production in lactating cows (Dohme et al., 2001, Dohme et al., 2004, Martin et al., 2008).

Diets were fed as TMR twice daily in equal portions before milking. For the first 10 d of the treatment period, diets were supplied ad libitum, after which feed intake was restricted to 95% of the DMI of the cow within a pair consuming the lowest amount of feed.

Milk Production and Composition

Milk yield was recorded daily in both experiments. In experiment 1, milk composition

was calculated as the weighted average of the respective analyzed composition and milk yield of 4 milkings of d 19 and 20. For experiment 2, the weighted averages of the respective analyzed composition and milk yield of 4 milkings of d 27, 28, 55 and 56 were used. Fat, protein, and lactose contents were determined according to ISO 9622 (ISO, 1999c), and MUN was determined using the pH difference technique (ISO 14637; ISO, 2004). Samples for analyses of milk fatty acid composition were collected on the same days, pooled, and analyzed according to the methods described by Vlaeminck et al. (2005) for experiment 1 and according to the methods of van Knegsel et al. (2007) for experiment 2.

Sampling and Analyses Procedures

Samples of TMR (approximately 500 g) were obtained daily when fresh TMR was prepared. These samples were subsequently pooled per period and treatment and subsampled for analyses. Orts, when present, were collected and pooled per period and subsequently subsampled for analyses. Samples of TMR and orts were frozen (-20°C) pending further analyses. Prior to analysis, samples were thawed and ground to pass a 1-mm screen.

During the measurement period in the respiration chambers, feces and urine were quantitatively collected, weighed, mixed thoroughly and subsampled for analysis of gross energy (GE), DM, ash, N, crude fat, and NDF. Samples were frozen pending analyses, thawed, and ground to pass a 1-mm screen before analyses.

Gross energy was determined using bomb calorimetry (IKA-C700, Janke & Kunkel, Heitersheim, Germany). Dry matter content was determined by drying at 103°C (ISO 6496; ISO 1999b) and ash content was determined by combustion at 550°C (ISO 5984; ISO 2002). The Kjeldahl method (ISO 5983; ISO 1997) was used to determine N content and CP was calculated by multiplication of total N content by 6.25. The crude fat content was analyzed using the Berntrop method (ISO 6492; ISO 1999a). Neutral detergent fiber was analyzed according to Van Soest et al. (1991), after pretreatment with amylase. The NDF contents reported include residual ash. Contents of ADF and acid detergent lignin were determined according to Van Soest (1973). The method of Ewers (ISO 6493: ISO, 2000) was used for determination of starch content and sugar content was determined by ethanol (40% v/v) extraction and subsequent titrimetric determination of reducing sugars (as glucose) according to the Luff-Schoorl method (NEN 3571; NEN, 1974).

Because urine does not contain crude fat, NDF or starch, contents of these components in the mixed samples of urine and feces were used for the determination of total tract apparent digestibility of crude fat, NDF and starch.

Evaluation of Rumen Fermentation Parameters

Rumen fluid samples (200 mL) in experiment 2 were collected 2 h post-feeding on d 27 and 28 of each period. Samples were obtained with a rigid polyvinylchloride (PVC) tube, which was perforated at the end (2-mm holes) to allow the rumen fluid to enter the tube. A piece of plastic tubing was inserted into the PVC tube and by application of a

vacuum, rumen fluid was aspirated from the rumen. After collection, sample containers were immersed in a bucket of ice water to stop microbial fermentation and samples were directly frozen after all samples had been taken.

Manual Protozoa Counting

Rumen fluid samples (250 mL) were obtained on d 5, 21 and 28 of each period in Experiment 2, 30 min after feeding. Protozoa counts were performed manually according to the method described by Dehority (1993). Briefly, a sample of rumen fluid was strained through cheesecloth and a 10-mL aliquot of strained rumen fluid was mixed with an equal volume of a 50% formalin solution in a graduated cylinder. Three replicate 1-mL aliquots of the mixture were transferred to test tubes and two drops of brilliant green dye were added to each of the test tubes. The solution was well mixed and allowed to stand overnight. The next day 9 mL of a 30% glycerol solution was added to each of the test tubes. The contents were well mixed and a 1-mL sample was transferred to a Rafter Counting Chamber No.1 (Pyser-SGI Ltd., Edenbridge, UK). Protozoa were counted under a microscope (magnification 100x), by counting 2 x 20 grids. This procedure was repeated with a second sample. When counts differed by no more than 10%, the average of both counts was accepted. A deviation larger than 10% resulted in additional counting of both samples in a different counting chamber. The number of protozoa was then calculated from the overall average of the 4 counts.

VFA and NH_3 Analyses

Analyses of VFA were performed according to the methods described by Van Nevel and Demeyer (1977). Rumen ammonia concentrations were determined according to the methods of Voigt and Steger (1967). Ammonia was flushed out with K_2CO_3 according to the microdiffusion method of Conway and O'Malley (1942) and subsequently captured in a boric acid solution and titrated with 0.01 M HCl. The average of the 2 samples taken on subsequent days for each animal was used for further calculations.

Statistical Analyses

Data were analyzed using the MIXED procedure in Genstat (11th edition, Lawes Agricultural Trust, Rothamsted, UK). In experiment 1, all parameters related to energy balance were averaged over the 7-d measurement period, expressed per kg $BW^{0.75}$ per day, subjected to ANOVA, including dietary treatment (CON vs. ADD) as fixed, and experimental replicate (i=1,.., 5) as random factor. In experiment 2, all data were averaged over the last 7 d of each period and subjected to ANOVA, including dietary treatment as a fixed factor and cow and period as random factors. Protozoa counts were subjected to repeated measures ANOVA to take repeated samples within the same animal into account. Statistical significance was declared at $P < 0.05$ and $P < 0.10$ indicated a noteworthy trend.

RESULTS

Feed Composition and Animal Performance

The chemical composition of the TMR for both treatments is shown in Table 2.1. As a result of restricted feeding, DMI was similar between treatments in both experiments (*Table 2.2*). Milk production tended to be lower for ADD in experiment 1, but milk production was unaffected by treatment in experiment 2. Milk fat content tended to be lower for ADD in experiment 1 and was decreased in experiment 2. Fat and protein corrected milk production for ADD was lower in experiment 1. Total daily milk fat yield was lower for the ADD groups in both experiments. Feeding ADD resulted in a lower MUN content (Expt. 1).

Table 2.1

Feedstuff and chemical composition of TMR containing a rumen inert fat source (control, CON) or linseed oil, lauric acid, myristic acid and Ca-fumarate (ADD), used in experiments 1 and 2

	Diet	
	CON	ADD
Item		
Grass silage, g/kg DM	290	288
Maize silage, g/kg DM	217	216
Barley straw, g/kg DM	18	18
Concentrates[1], g/kg DM	439	436
Fractionated palmitic acids (C16:0) , g/kg DM	31	
Lauric acid (C12:0) , g/kg DM		4
Myristic acid (C14:0) , g/kg DM		12
Linseed oil, g/kg DM		15
Limestone, g/kg DM	5	
Calcium fumarate, g/kg DM		7
Vitamin E, g/kg DM		4
GE, MJ/kg DM	19.4	19.5
DM, g/kg	588	580
Crude ash, g/kg DM	79	78
Crude protein, g/kg DM	170	170
NDF, g/kg DM	349	339
Crude fat, g/kg DM	64	62
Starch, g/kg DM	168	165
Sugar, g/kg DM	69	70

[1] Concentrates were composed of 22.2% maize, 20.0% hominy feed, 15.0% dry beet pulp, 12.5% maize gluten feed, 10.9% barley, 10.0% formaldehyde-treated soybeanmeal, 6.2% soybean meal, 1.5% cane molasses, 1.0% mineral premix, 0.5% NaCl and 0.2% limestone on a product basis

Table 2.2

Milk production, milk composition and DMI of dairy cows fed a control diet (CON) containing a rumen inert fat source or a diet containing a mixture of linseed oil, lauric acid, myristic acid, and Ca-fumarate (ADD)

	Experiment 1 *(n = 10/treatment)*				Experiment 2 *(n = 6/treatment)*			
	CON	ADD	SEM	P-value	CON	ADD	SEM	P-value
DMI, kg/d	16.4	15.9	0.21	0.173	20.0	19.8	0.22	0.544
Milk production, kg/d	28.9	26.1	0.86	0.053	32.0	33.2	0.59	0.175
FPCM[1], kg/d	30.5	26.3	0.70	0.002	33.4	31.6	0.754	0.124
Milk fat content, %	4.63	4.10	0.193	0.087	4.38	3.63	0.154	0.006
Milk protein content, %	3.27	3.35	0.079	0.533	3.54	3.47	0.075	0.539
Milk lactose content, %	4.61	4.53	0.037	0.172	4.36	4.38	0.052	0.881
MUN, mg/dL	10.1	7.9	0.54	0.019	11.6	10.3	0.46	0.064
SCC, x1000 cells/mL	123	208	77.7	0.458	136	169	20.3	0.271
Fat yield, g/d	1313	1079	38.7	0.002	1374	1176	48.8	0.017
Protein yield, g/d	932	882	20.5	0.115	1116	1131	23.6	0.654

[1] Fat- and protein-corrected milk.

Methane Production

Methane production expressed in grams per day was 10% lower in experiment 1 for ADD ($P < 0.05$, *Table 2.3*). When expressed relative to gross energy intake (GEI), methane production was also lower for ADD compared with CON (5.7 and 6.2% of GEI, respectively). Due to a lower production of milk for ADD in Expt. 1, methane expressed per kilogram of milk was not different between treatments.

Table 2.3

Methane (CH_4) production from dairy cows fed a control diet (CON) containing a rumen inert fat source or a diet containing a mixture of linseed oil, lauric acid, myristic acid and Ca fumarate (ADD), n = 5 per treatment

	Diet			
	CON	ADD	SEM	P-value
Item				
CH_4, g/day	362	325	7.1	0.021
CH_4, g/kg DMI	22.1	20.5	0.65	0.146
CH_4, % of GEI[1]	6.2	5.7	0.10	0.025
CH_4, g/kg milk	12.8	12.7	0.43	0.906

[1] Gross energy intake

Apparent Digestibility and Energy Balance

Apparent digestibility of NDF and starch was unaffected by treatment (*Table 2.4*). Apparent fat digestibility was higher for ADD. No differences in GEI, metabolizable energy intake or energy retention between treatments were found (*Table 2.4*). Cows on both treatments mobilized body reserves during the experimental period despite the fact that they were already in the advanced stages of lactation. The amount of energy lost as methane was lower for the ADD-fed cows. Cows receiving the ADD treatment partitioned less energy to milk production, which was reflected numerically in the overall energy balance.

Rumen Fermentation Profile

The total count of rumen protozoa tended to be lower for treatment ADD in experiment 2 (*Table 2.5*). This decrease was especially apparent on d 5 of the experimental treatment, whereas the decrease was only numerical on d 21 and 28.

Concentrations of ruminal VFA were lower in cows on the ADD treatment. Within the VFA profile, the propionate proportion increased for the ADD treatment, whereas proportions of acetate and butyrate decreased, resulting in lower acetate-to-propionate ratio for the ADD treatment. Ammonia concentrations in the rumen fluid were lower for the ADD treatment.

Table 2.4

Energy balance and total tract apparent nutrient digestibility in dairy cows fed a control diet (CON) containing a rumen inert fat source or a diet containing a mixture of linseed oil, lauric acid, myristic acid, and Ca-fumarate (ADD), n = 5 per treatment

	Diet			
	CON	ADD	SEM	P-value
Item				
Metabolic BW, $kg^{0.75}$	113.3	114.6	1.36	0.547
Gross energy intake, kJ/kg of $BW^{0.75}$ per d	2852	2757	61.5	0.339
ME intake[1], kJ/kg of $BW^{0.75}$per d	1759	1737	59.0	0.800
ME:GE ratio[2], %	61.8	62.9	0.94	0.566
Methane production, kJ/kg of $BW^{0.75}$per d	177.1	157.3	3.46	0.015
Heat production, kJ/kg of $BW^{0.75}$per d	1014	1016	12.03	0.901
Energy in milk, kJ/kg of $BW^{0.75}$per d	913	786	17.5	0.007
Energy retention total[3], kJ/kg of $BW^{0.75}$per d	-167	-65	45.9	0.189
Energy retention protein[4], kJ/kg of $BW^{0.75}$per d	8.7	28.6	8.62	0.178
Energy retention fat[5], kJ/kg of $BW^{0.75}$per d	-176	-93	41.1	0.228
NDF digestibility, %	69.6	68.1	0.68	0.173
Starch digestibility, %	97.5	97.8	0.15	0.194
Fat digestibility, %	58.1	69.4	1.20	0.003

[1] ME intake = gross energy intake (GEI) – methane production – energy in feces + urine.
[2] GE = gross energy.
[3] Energy retention total = MEI – heat production – energy in milk.
[4] Energy retention protein = protein gain * 23.6 kJ/g.
[5] Energy retention fat = energy retention total – energy retention protein.

Table 2.5

Volatile fatty acid, ammonia concentrations and protozoa numbers in rumen fluid taken 2 h after feeding of fistulated dairy cows fed a control diet (CON) containing a rumen inert fat source or a diet containing a mixture of linseed oil, lauric acid, myristic acid, and Ca-fumarate (ADD), n = 5/treatment

	Diet			
	CON	ADD	SEM	P-value
Item				
VFA concentration, mmol/L	134.9	126.3	2.28	0.029
Acetate, % of VFA	60.3	58.6	0.32	0.006
Propionate, % of VFA	19.8	23.0	0.40	< 0.001
Butyrate, % of VFA	15.3	13.6	0.39	0.015
Isobutyrate, % of VFA	1.1	1.0	0.05	0.108
Valerate, % of VFA	1.6	1.8	0.09	0.205
Isovalerate, % of VFA	1.9	2.0	0.08	0.210
Acetate: propionate ratio	3.1	2.6	0.06	< 0.001
NH_3, mg/100 mL	36.6	30.7	1.16	0.007
Protozoa count d 5, x 10^5/mL	2.60	1.73	0.191	0.012
Protozoa count d 21, x 10^5/mL	2.64	1.75	0.343	0.105
Protozoa count d 28, x 10^5/mL	2.66	2.25	0.177	0.139
Protozoa count average[1], x 10^5/mL	2.63	1.91	0.121	0.088

[1] P-value of repeated measures analysis, time effect P = 0.498, time x treatment P = 0.586

Milk Fatty Acid Composition

The dietary treatments were clearly reflected in the milk fatty acid composition (*Table 2.6*). Concentrations of C12:0 and C14:0 in milk fat increased in both experiments as a result of the addition of these fatty acids to the ADD diet. The content of C16:0 in milk fat was higher for the CON treatment in both experiments, reflecting the addition of this fatty acid to the CON diet. The content of C16:1c9 was also higher in the CON diet in both experiments.

Clear shifts in odd and branched-chain fatty acids (OBCFA) were observed as a result of the dietary treatment. Contents in milk fat of C15:0, iso C15:0 and anteiso C15:0 were all higher, and C17:0 and C17:1 cis-9 lower, with the ADD diet. Most shifts were consistent in both experiments. C18:1 cis-9 and C18:2 cis-9,12 were higher with the CON diet, whereas C18:1 trans-10+11 was higher with the ADD diet.

Table 2.6

Milk fatty acid composition (g/100 g fatty acids) of milk from dairy cows fed a control diet (CON) containing a rumen inert fat source or a diet containing a mixture of linseed oil, lauric acid, myristic acid, and Ca-fumarate (ADD)

	Experiment 1 *(n = 10/treatment)*				Experiment 2 *(n = 6/treatment)*			
	CON	ADD	SEM	P-value	CON	ADD	SEM	P-value
C 4:0	2.99	2.80	0.117	0.277	3.48	3.41	0.145	0.715
C 6:0	1.85	1.65	0.073	0.079	1.80	1.68	0.095	0.390
C 8:0	1.06	0.95	0.045	0.130	0.88	0.85	0.050	0.693
C 10:0	2.30	2.11	0.098	0.206	1.78	1.77	0.103	0.950
C 12:0	2.58	3.73	0.083	< 0.001	2.10	3.14	0.069	< 0.001
C 14:0	9.64	17.4	0.247	< 0.001	8.25	15.3	0.259	< 0.001
C 14:1 cis-9	0.78	1.34	0.182	0.059	1.00	2.01	0.076	< 0.001
C 16:0	35.3	22.6	0.478	< 0.001	36.5	23.5	0.345	< 0.001
C 16:1 cis-9	2.03	1.41	0.122	0.006	2.30	1.61	0.053	< 0.001
C 15:0	0.79	0.92	0.035	0.028	0.78	0.90	0.024	0.004
C 15:0 iso	0.17	0.20	0.005	0.003	0.14	0.17	0.004	0.002
C 15:0 anteiso	0.37	0.47	0.021	0.009	0.36	0.42	0.007	< 0.001
C 17:0	0.49	0.42	0.014	0.009	0.39	0.35	0.011	0.038
C17:0 iso	0.31	0.34	0.014	0.127	0.19	0.20	0.007	0.639
C17:0 anteiso	0.22	0.14	0.024	0.029	0.39	0.41	0.009	0.107
C 17:1 cis-9	0.09	0.08	0.017	0.835	0.21	0.16	0.008	0.002
C 18:0	10.1	10.1	0.374	0.935	7.67	8.26	0.283	0.168
C 18:1 cis-9	19.1	16.9	0.509	0.011	20.1	18.2	0.376	0.004
C 18:1 trans-10+11	0.99	2.13	0.223	0.006	1.24	2.48	0.340	0.028
C 18:2 cis- 9,12	1.63	1.39	0.043	0.003	1.65	1.38	0.052	0.004
C 18:3 cis-9,12,15	0.39	0.47	0.034	0.143	0.47	0.51	0.015	0.068
C 20:0	0.09	0.13	0.017	0.107	0.12	0.11	0.002	0.001
C 20:1 cis-9	0.19	0.34	0.045	0.044	0.12	0.11	0.006	0.152

DISCUSSION

Effects of the Additives on Enteric Methane Production

The rationale behind the use of a mixture of feed additives was to make use of the known methane-suppressing properties of medium-chain fatty acids (MCFA) and linseed oil and to prevent accumulation of H by providing an alternative H sink in the form of Ca fumarate. The mixture of feed additives successfully lowered methane emissions from lactating dairy cows in this experiment.

Results of experiments investigating the methane-mitigating effect of dietary fumarate are equivocal, but in situations of an increased concentration of H in the rumen, fumarate may be an effective hydrogen sink (Ungerfeld et al., 2007). In 2 experiments, in which fumarate or fumaric acid was fed to ruminants, decreases in methane production were observed (Bayaru et al., 2001, Wallace et al., 2006). However, other groups reported no effects on methane emission when fumarate was fed (Beauchemin and McGinn, 2006, Kolver and Aspin, 2006, Molano et al., 2008). In the current experiment, 116 g of Ca fumarate was fed per animal per day. If all Ca fumarate fed to the animal would have been converted to propionate in the rumen, methane production would have decreased by 4.9 g of methane and the maximum theoretical decrease in methane production thus attributable to the addition of Ca fumarate in these diets could only account for little over 1% of total methane emissions. For this experiment, we can probably assume that effects of Ca fumarate addition were negligible and the decrease in methane emitted can be ascribed to the fatty acids and oils used.

Addition of dietary fats and oils, the main components of the mixture, has been shown to lower methane emissions from ruminants in numerous studies (Machmüller et al., 2003, Jordan et al., 2006b, Martin et al., 2008). Martin et al. (2008) demonstrated a 64% reduction in daily methane production, determined with the SF_6-technique, when linseed oil (5.8% of diet DM) was dosed orally to lactating dairy cows twice daily. This decrease was hypothesized to result from a decrease in total tract NDF digestibility (-11%). Although DMI decreased (-26%), methane production per kilogram OM intake decreased as well (-52%). Martin et al. (2008) hypothesized that, in their experiment, NDF digestibility was considerably depressed in the rumen and that this was partly compensated for by fermentation in the hindgut. Because the rumen is the main site of methanogenesis, this could explain the major reduction in methane production. In the present study, linseed oil was fed at a much lower level (1.5% of DM) and was mixed in the TMR, resulting in a much more gradual intake along the day. Total-tract NDF digestibility was not affected in the present experiment, but it is possible that ruminal NDF fermentation was decreased in cows fed the ADD diet and that this was completely compensated for by increased NDF fermentation in the large intestine. Beauchemin et al. (2009) added 9.3% crushed linseed to the diet of lactating dairy cows and observed an 18% decrease in methane production in climate chambers without effects on DMI. However, when methane production was expressed per kilogram of digestible DM, no differences in methane production were observed. The decreased methane production reported by Beauchemin et al. (2009) thus appears to be completely attributable to a decreased DM digestibility. In the study of Beauchemin et al. (2009) the number of rumen protozoa was suppressed when cows were fed crushed linseed. In the present experiment the number of rumen protozoa also tended to be lower for ADD, which may explain the decreased methane production observed. Defaunation has been associated with lower methanogenesis due to the symbiotic relationship between methanogens and protozoa (Hegarty, 1999).

Odongo et al. (2007b) observed a 36% decrease in daily methane production when myristic acid was added to the diets of lactating dairy cows at 5% of diet DM. Dry matter intake tended to be lower for the myristic acid-supplemented cows. Dohme et al. (2004)

fed diets containing either C12:0, C14:0, or C18:0 to dairy cows (5% of DM). Methane production was not affected by the inclusion of C14:0, but was decreased by 21% for the C12:0 diet compared with the C18:0 diet. However, the inclusion of C12:0 also suppressed DMI and when methane production was expressed per kilogram of DMI, it was no longer different from the control. Jordan et al. (2006a) fed increasing quantities of coconut oil (125, 250, and 375 g/d; 1.3, 2.7 and 4.6% of DM, consisting predominantly of C12:0 and C14:0) to beef heifers. Daily methane emissions were decreased (13, 20 and 39% respectively), but DMI also linearly decreased with increasing concentrations of coconut oil in the diet. Dry matter digestibility also decreased linearly with increasing dose of coconut oil, probably reflecting a dose-dependent decrease in NDF digestibility. In another experiment by Jordan et al. (2006b), 250 g of refined coconut oil (equivalent to 2.7% of DM) was fed to beef heifers on a daily basis. Methane production was decreased by 19%, but digestibility coefficients were not affected. However, total ruminal VFA concentrations were suppressed and the total number of protozoa was reduced by 63%.

The 10% decrease in methane production achieved in our study appears rather modest when compared with other studies (Jordan et al., 2006b, Odongo et al., 2007b, Martin et al., 2008, Beauchemin et al., 2009). However, the methane decreases achieved in the other studies were, at least in part, related to decreases in DMI (Beauchemin et al., 2009), a difference in fat content between the control ration and the treatment ration (Jordan et al., 2006b) or both (Jordan et al., 2006a, Martin et al., 2008). Dry matter intake is one of the main drivers of the quantity of methane produced (Ellis et al., 2009) and differences in DMI between treatments as a consequence of the added oil and fat will cause an indirect effect on methane production. Differences in fat content between the control ration and the treatment ration will inherently lead to differences in dietary FOM content and this in itself will also lead to differences in the quantity of methane produced. In this experiment we excluded differences in DMI and in dietary fat content as factors indirectly influencing methane emissions.

Because DMI and dietary fat content did not differ, results of the present experiment show that the addition of the products used in this experiment have an effect on methane emissions related to causal factors other than indirect effects on DMI and differences in dietary fat content. This additional effect might be related to specific effects of the fats and oils on the rumen microbial consortium (Dohme et al., 2001, Zhang et al., 2008).

Possible Mode of Action of Methane Decrease

The ruminal concentration of VFA was suppressed for the ADD treatment, which may indicate a lower level of rumen fermentation or increased absorption of VFA from the rumen. The overall decrease in rumen fermentation may provide a partial explanation for the observed lower methane production. Dietary fat addition is known to lower rumen degradation of carbohydrates, specifically structural carbohydrates. This negative effect is more pronounced when linseed oil is added to the diet compared with saturated fats (Doreau and Chilliard, 1997).

Within the VFA, the proportion of propionate increased, possibly reflecting decreased structural carbohydrate degradation. Indeed, the fatty acids and linseed oil used in

this study have been shown to decrease structural carbohydrate degradation in other studies (Jordan et al., 2006a, Martin et al., 2008). A shift towards propionate-orientated fermentation is commonly observed when feeding oils to dairy cows (McGinn et al., 2004, Beauchemin and McGinn, 2006). This increased propionogenesis may also result from an increased H pressure in the rumen as a consequence of the decreased methanogenesis. Total tract apparent digestibility of NDF was not affected by treatment, and decreased structural carbohydrate degradation, thus, does not appear to offer an explanation for the observed decrease in methane emission. It could, however, be possible that rumen fermentation of the NDF fraction was negatively affected by the inclusion of fats and oils in the ADD diet, but that this was compensated for by fermentation in the large intestine. The compensatory fermentation in the hindgut would yield fewer nutrients for the dairy cow and could possibly explain the lower milk production observed on the ADD treatment. The fermentation of NDF in the large intestine could also yield less methane per unit of NDF fermented (Immig, 1996), due to the possibility of reductive acetogenesis in the hindgut.

Protozoa numbers tended to be lower in the ADD treatment. Defaunation has been associated with lower methanogenesis due to the symbiotic relationship between methanogens and protozoa (Hegarty, 1999). Defaunated animals often have a higher ruminal propionate concentration (Bird et al., 2008) and lower rumen NH_3 levels in rumen fluid (Eugène et al., 2004), which was also observed in this experiment. Thus, the inhibitory effect of the fats and oils added on the rumen protozoa may be another explanation for the observed methane reduction. Partial defaunation has been observed before when feeding MCFA (Lovett et al., 2003, Hristov et al., 2004) or linseed oil (Broudiscou et al., 1994, Zhang et al., 2008) to ruminants.

Changes in Milk Composition

Milk fat content was lower for the ADD group. A decrease in milk fat content has been observed before when feeding linseed oil to dairy cows (e.g. Martin et al., 2008) and results from the accumulation of products of incomplete ruminal biohydrogenation of the unsaturated fatty acids in linseed oil (Bauman and Griinari, 2003). The lower milk fat content on this treatment can be explained by the inhibition of mammary lipogenesis by these intermediates. Milk urea nitrogen was lower for the ADD treatment, possibly reflecting a decreased formation of rumen ammonia as a result of defaunation (Eugène et al., 2004). In experiment 2, ruminal ammonia concentrations and protozoal numbers were in fact lower for the ADD treatment.

Vlaeminck and Fievez (2005) developed equations to predict the quantity of methane produced by dairy cows from the proportion of odd and branched-chain fatty acids in milk. They predicted that iso C15:0 was positively, and C15:0 negatively related to methanogenesis. Our data however, indicate C15:0 and iso C15:0 to be negatively associated with methane production. In recent work by Chilliard et al. (2009) C15:0 was positively correlated with methane production. The concentration of iso C15:0 was not found to be related to methanogenesis in that experiment. Vlaeminck and Fievez (2005) explained the relationship they observed by a higher enrichment of iso C15:0 in H-producing bacteria, whereas propionate producing bacteria contained a relatively high

proportion of C15:0. In experiment 2, C15:0 concentrations increased by approximately 15%, suggesting an increase in propionate-producing bacteria. This was also confirmed by the higher proportion of propionate in rumen fluid. However, iso C15:0 concentrations in milk also increased by 18 to 21% when the ADD ration was fed. This suggests an increase in H-producing bacteria. The equation predicted almost equal methane production in both treatments in this experiment, whereas in reality, a 10% decrease was observed. However, Vlaeminck and Fievez (2005) expressed methane proportionally to the VFA concentration (CH_4/VFA); in this experiment methane production was 10% lower for the ADD treatment, but VFA production was also 6% lower for this treatment. Expressing methane production proportionally to VFA in this experiment would lead to similar values for both treatments.

CONCLUSIONS

The diet containing a mixture of C12:0, C14:0, linseed oil, and Ca fumarate successfully lowered daily methane emissions from dairy cows, when compared with an isolipidic control diet fed at similar DMI. However, methane emissions per kilogram of FPCM were not affected by feeding the mixture. Earlier reported methane decreases for these fats and oils appear to be largely caused by indirect methane decreases as a consequence of lower DMI and different fat contents between diets.

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CHAPTER 3

Dietary inclusion of diallyl disulfide, yucca powder, calcium fumarate, an extruded linseed product, or medium-chain fatty acids does not affect methane production in lactating dairy cows

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ABSTRACT

Two similar experiments were conducted to assess the effect of diallyl disulfide (DADS), yucca powder (YP), calcium fumarate (CAFU), an extruded linseed product (UNSAT) or a mixture of capric and caprylic acid (MCFA) on methane production, energy balance and dairy cow performance. In experiment 1, a control diet (CON1) and diets supplemented with 56 mg of DADS/kg of dry matter (DM), 3 g of YP/kg of DM or 25 g of CAFU/kg of DM were evaluated. In experiment 2, an inert saturated fat source in the control diet (CON2) was exchanged isolipidically for an extruded linseed source (100 g/kg of DM; UNSAT) or a mixture of C8:0 and C10:0 (MCFA; 20.3 g/kg of DM). In experiment 2, a higher inclusion level of DADS (200 mg/kg of DM) was also tested. Both experiments were conducted using 40 lactating Holstein-Friesian dairy cows. Cows were adapted to the diet for 12 d and were subsequently kept in respiration chambers for 5 d to evaluate methane production, diet digestibility, energy balance and animal performance. Feed intake was restricted to avoid confounding effects of possible differences in ad libitum feed intake on methane production. Feed intake was, on average, 17.5 and 16.6 kg DM/d in experiments 1 and 2, respectively. None of the additives reduced methane production in vivo. Methane production in experiment 1 was 450, 453, 446 and 423 g/d for CON1 and the diets supplemented with DADS, YP and CAFU, respectively. In experiment 2, methane production was 371, 394, 388 and 386 g/d for CON2 and the diets supplemented with UNSAT, MCFA and DADS, respectively. No effects of the additives on energy balance or neutral detergent fiber digestibility were observed. The addition of MCFA increased milk fat content (5.38% vs. 4.82% for control) and fat digestibility (78.5% vs. 59.8% for control), but did not affect milk yield or other milk components. The other products did not affect milk yield or composition. Results from these experiments emphasize the need to confirm methane reductions observed in vitro with in vivo data.

INTRODUCTION

The global dairy sector is estimated to contribute 4.0% of global anthropogenic greenhouse gases, with the majority of these gases produced on the dairy farm (FAO, 2010). Enteric methane emissions account for 52% of the total amount of greenhouse gases produced during milk production and processing (FAO, 2010). Dietary strategies can influence the amount of enteric methane produced by dairy cows (Beauchemin et al., 2008, Ellis et al., 2008), and the reduction of enteric methane production has become an important goal in ruminant nutrition research.

Diallyl disulfide (DADS), one of the main components of garlic oil, has been shown to decrease methane production in vitro up by up to 69% (Busquet et al., 2005b, Macheboeuf et al., 2006). It is thought to act through a direct effect on the enzyme system of the methanogenic archaea, inhibiting their activity (Busquet et al., 2005a). Yucca extract has been shown to decrease the number of rumen protozoa when fed to dairy cows (Lovett et al., 2006) or heifers (Hristov et al., 1999). Some of the rumen methanogens live in close association with the protozoa (Newbold et al., 1995, Hegarty, 1999) and yucca extract has been demonstrated to lower methane production in vitro (Lila et al., 2003). Fumarate is a precursor of propionate in the rumen. Propionogenesis from fumarate consumes hydrogen, thus lowering hydrogen availability for methanogenesis (Wallace et al., 2006), and methane reduction as a consequence of fumarate addition has been demonstrated in vitro (Asanuma et al., 1999). Responses of in vivo methane production to dietary fumarate have been equivocal (Bayaru et al., 2001; Kolver and Aspin, 2006; Wallace et al., 2006; McCourt et al., 2008).

The methane-depressing effects of DADS and yucca powder had not been confirmed at the time of the implementation of experiment 1. Experiment 1 was designed to test the effect of DADS, yucca powder and calcium fumarate on methane production by lactating dairy cows. We hypothesized, based on previous in vitro results, that these compounds would lower methanogenesis in the lactating dairy cow.

Addition of fat to ruminant diets is frequently proposed as a strategy to lower methanogenesis (Eugene et al., 2008). However, different fatty acids have different effects on methanogenesis (Czerkawski et al., 1966b, Prins et al., 1972). For the C18 fatty acids, inhibition of methane production appears to increase with the degree of unsaturation (Czerkawski et al., 1966a). Specific medium-chain fatty acids have been found to lower methanogenesis in vitro (Dohme et al., 2001). Ajisaka et al. (2002) observed significant methane reductions when cyclodextrin complexes of caprylic (C8:0) or capric (C10:0) acid were incubated with rumen fluid in vitro, but we are not aware of any in vivo evaluations of these fatty acids. In experiment 2 we exchanged a saturated fat source (containing mainly C16:0) for a fat source containing C8:0 and C10:0 or a source containing extruded linseed (rich in C18:2 and C18:3), to assess the methane-lowering capacity of these specific fatty acids. Diets in experiment 2 were isolipidic to avoid effects of dietary fat content on methane production. We hypothesized that methane production would be lower for sources rich in C8:0 and C10:0 or C18:2 and C18:3 compared with the source supplying mainly C16:0.

In both experiments, indirect effects of level of feed intake on methane production were avoided by restricting the amount of feed offered. A reduction of methane emission may lead to increased milk energy output or to an improved energy balance, provided that the extent of fermentation is not affected. To verify this, energy balances were determined in both experiments.

MATERIAL AND METHODS

Experimental Procedures

Two completely randomized block experiments (Exp.) were conducted, each with 4 treatments and 10 lactating Holstein-Friesian dairy cows per treatment. Cows were blocked on fat- and protein-corrected milk production, parity, and DIM before the experiment (10 blocks of 4 animals in each experiment). Within each block, cows were randomly assigned to 1 of 4 dietary treatments. Cows in Exp. 1 produced 29.8 ± 5.7 kg of milk/d and were 97 ± 70 DIM at the start of the experiment. In Exp. 2, cows produced 27.9 ± 7.0 kg of milk/d and were 167 ± 99 DIM. In Exp. 1, dietary treatments consisted of a control diet (CON1) and diets supplemented with 56 mg DADS/kg of DM (DADS1), 3 g/kg of DM yucca powder (YP) or 25 g/kg of DM calcium fumarate (CAFU). In Exp. 2, a rumen-inert fat source (CON2) was replaced isolipidically by an extruded linseed product (UNSAT; 100 g/kg of DM) or a source containing C8:0 and C10:0 fatty acids (MCFA; 20.3 g/kg of DM). In Exp. 2 DADS was again evaluated (DADS2) but at an inclusion rate of 200 mg/kg of DM.

Source of Test Products

Diallyl disulfide (Vetcare PVT, Bangalore, India) in liquid form was applied to a silica carrier (Provimi France, Treize Vents, France) to produce a solid material containing 10% DADS. Yucca powder (Yucca-Plus Powder, Agroin, Ensenada, Mexico) was purchased from Jadis Additiva (Schiedam, the Netherlands). Calcium fumarate was supplied by Kemin Industries Inc. (Herentals, Belgium). The extruded linseed product (Promax 20/20, Provimi France) consisted of 50% extruded linseed, 2% rapeseed, 18% sunflower meal and 30% wheat bran. The C8/C10 product was produced by applying a mixture of liquid C8:0 and C10:0 (Aveve, Leuven, Belgium) to a silica carrier (Provimi France) to provide a material containing 45% fatty acids and 55% carrier material.

Housing

The Animal Care and Use Committee of Wageningen University (Wageningen, the Netherlands) approved the experimental protocols of both experiments. Animals were housed in the facilities of Wageningen University and Research Centre. Cows were individually housed in tie-stalls and milked twice daily at 0600 and 1700 h. Animals remained in tie-stalls for 12 d to become accustomed to the diet and restriction in movement. After this period, animals were housed in 1 of 2 identical respiration chambers to determine gaseous exchange, energy balance and diet digestibility. Because 2 chambers were available, measurements were obtained in 10 periods, staggered in time. Within each

period, 2 cows receiving the same treatment were housed in one chamber, and 2 cows receiving a different treatment were housed in the other chamber. Within each chamber, the 2 cows originated from a different block. The experimental unit for data measured in the respiration chambers (e.g., methane production, diet digestibility parameters) therefore consisted of a pair of cows. The respiration chambers have been described in detail by Verstegen et al. (1987). Cows remained in the respiration chambers for a period of 5 d. After completion of the 5-d measurement period, feces, urine and cleaning water were quantitatively collected, weighed, and subsampled for determination of NDF and crude fat. Both NDF and crude fat were assumed to be absent in urine, allowing for calculation of digestibility of these components from analyses of NDF and crude fat in the combined mixture of feces and urine.

Diets and Feeding

Cows in Exp. 1 were fed a diet consisting of 40% grass silage, 26% corn silage, and 34% concentrates on a DM basis. The concentrates consisted of 30.0% soybean meal, 24.1% wheat, 18.1% corn, 12.1% dried beet pulp, 12.1% rapeseed meal, 1.2% limestone, and 2.4% of a mineral premix. The additives were hand-mixed into the diet at the time of feeding. Because their inclusion rate was low, this did not affect the average chemical composition of the TMR. The chemical compositions of the TMR used in both experiments are shown in Table 3.1.

Cows in Exp. 2 were fed a TMR containing 41% grass silage, 35% corn silage, 14% concentrates, and 10% of a mixture containing the experimental test products on a DM basis. The concentrates consisted of 52.1% soybean meal, 38.2% wheat, 5.2% limestone and 4.5% of a mineral premix. The experimental test products were included in a mixture that was hand-mixed into the TMR at the time of feeding. The composition of these mixtures is shown in Table 3.2.

Animals in both experiments were fed equal portions twice daily during milking. Diets were supplied individually and were supplied ad libitum for the first 8 d in the tie-stalls. From d 8 to 17, feed intake was restricted per block to 95% of the ad libitum feed intake of the animal consuming the lowest amount of feed during d 5 to 8 within a block. In the respiration chambers, orts were collected when present, pooled per cow and period, and frozen pending analyses.

Sampling and Chemical Analyses

Milk yield was recorded during each milking. During the period in the respiration chambers, 2 representative samples (3 g/kg milk for each sample) were obtained at each milking for each cow. These samples were pooled per cow for the entire period. Milk was analyzed for fat, protein and lactose content according to ISO 9622 (ISO, 1999c) and the MUN content was determined employing the pH difference technique (ISO 14637; ISO, 2004). Gross energy content was determined using bomb calorimetry (IKA-C700, Janke & Kunkel, Heitersheim, Germany) and the N content of milk was determined according to Kjeldahl analysis.

Feed was sampled (±500 g) directly after preparation, before inclusion of the additives. Samples were stored frozen (-20°C) pending further analyses. At the end of the experiment, samples were pooled per period and analyzed for their chemical composition. In Exp. 2 samples, (±100 g) of the additive mixtures were taken weekly and stored frozen (-20°C) until analysis. Feces and urine were quantitatively collected over the entire measurement period, weighed, thoroughly mixed and subsampled for analyses. Prior to analysis, samples of feed and feces were freeze-dried and ground to pass a 1-mm screen. Dry matter, CP, crude fat, sugar, starch and NDF content of TMR, additive, and manure samples were determined according to the methods described in detail by Abrahamse et al. (2008).

Statistical Analyses

Data collected during the measurement period only were used for statistical analyses. Daily data were averaged per period before analysis. Data collected for pairs of cows (energy balance traits and diet digestibility) were subjected to ANOVA, with treatment and respiration chamber as fixed factors ($Y_{ij} = \mu_{ij} + \text{respiration chamber}_i + \text{treatment}_j + \varepsilon_{ij}$, in which Y_{ij} = observed response, μ_{ij} = overall mean, respiration chamber$_i$ = effect of respiration chamber i, treatment$_j$ = effect of treatment j, and ε_{ij} = residual error). As the 2 cows within a pair originated from a different block, block was not included in the statistical analysis of these traits. Assigning animals to treatments within a block served the purpose of minimizing the reduction in feed intake when feed intake was restricted. Data collected for individual cows (DMI, milk yield, and milk composition) were subjected to ANOVA, with block, treatment, and respiration chamber as fixed factors ($Y_{ijk} = \mu_{ijk} + \text{block}_i + \text{respiration chamber}_j + \text{treatment}_k + \varepsilon_{ijk}$, in which Y_{ijk} = observed response, μ_{ijk} = overall mean, block$_i$ = effect of block i, respiration chamber$_j$ = effect of respiration chamber j, treatment$_k$ = effect of treatment k, and ε_{ijk} = residual error). The effect of chamber was not significant for any of the parameters analyzed in both experiments. When the treatment effect was significant, treatment means were separated by means of Tukey's test. The statistical program Genstat (11th ed., Lawes Agricultural Trust, Rothamsted, UK) was used to analyze the results.

Results

Feed Composition and Animal Performance

The chemical compositions of the TMR used in Exp. 1 and Exp. 2 are shown in Table 3.1. The dietary additives used in Exp. 1 were manually mixed into this TMR. The ingredient and chemical composition of the mixtures including the dietary additives used in Exp. 2 are shown in Table 3.2.

Diets in both experiments had a comparable chemical composition, except for the level of crude fat, which was higher for Exp. 2 due to the addition of the fat-rich mixtures. The shift in fatty acid pattern of the diets was successfully established, with C16:0 being the most important fatty acid in CON2, C18:2 and C18:3 in UNSAT, and C8:0 and C10:0 in the MCFA treatment.

Table 3.1

Ingredient, analyzed chemical composition and fatty acid composition of TMR fed in Experiments 1 and 2[1]

	Exp. 1	Exp. 2			
Item	CON1	CON2	UNSAT	MCFA	DADS2
Grass silage (% of DM)	40	41	41	41	41
Corn silage (% of DM)	26	35	35	35	35
Concentrates (% of DM)	34	14	14	14	14
Additive mixture (% of DM)	-	10	10	10	10
DM (g/kg)	441	424	430	429	424
GE (MJ/kg of DM)	19.3	20.2	19.9	19.6	20.2
Crude ash (g/kg of DM)	76	77	78	94	78
CP (g/kg of DM)	167	165	163	159	165
NDF (g/kg of DM)	415	410	417	403	415
Crude fat (g/kg of DM)	33	58	55	58	60
C8:0[2] (g/kg of DM)	0.0	0.0	0.0	10.1	0.0
C10:0 (g/kg of DM)	0.0	0.0	0.0	10.1	0.0
C12:0 (g/kg of DM)	0.0	0.0	0.0	0.0	0.0
C14:0 (g/kg of DM)	0.0	0.3	0.0	0.0	0.3
C16:0 (g/kg of DM)	3.2	19.5	4.7	3.5	19.5
C16:1 (g/kg of DM)	0.2	0.2	0.2	0.2	0.2
C18:0 (g/kg of DM)	0.4	1.5	1.2	0.5	1.5
C18:1 (g/kg of DM)	3.6	4.9	5.9	2.7	4.9
C18:2 (g/kg of DM)	8.0	8.1	10.2	7.5	8.1
C18:3 (g/kg of DM)	5.2	8.2	17.3	7.4	8.2
> C20:0 (g/kg of DM)	0.2	0.3	0.1	0.1	0.3
Saturated fatty acids (S: g/kg of DM)	3.9	21.6	6.1	24.4	21.6
Unsaturated fatty acids (U: g/kg of DM)	17.0	21.4	33.7	17.7	21.4
U:S ratio	4.4	1.0	5.5	0.7	1.0

[1] CON1 = control diet in Exp. 1; CON2 = control diet in Exp. 2; UNSAT = rumen-inert fat source in CON2 was replaced isolipidically by an extruded linseed product; MCFA = rumen-inert fat source in CON2 was replaced isolipidically by a source containing C8:0 and C10:0 fatty acids; DADS2 = diet supplemented with 200 mg/kg DM of diallyl disulfide.

[2] Fatty acid profiles were calculated from CVB (2007).

Table 3.2

Ingredient and analyzed chemical composition of mixtures containing the dietary additives for experiment 2; mixtures were added to the TMR at 10% of DM

	Exp. 2 diet[1]			
	CON2	UNSAT	MCFA	DADS2
Item				
Ground wheat (g/kg of DM)	250		190	248
Mechanically extracted linseed meal (g/kg of DM)	550		360	550
Fractionated palm oil[2] (g/kg of DM)	200			200
DADS product[3] (g/kg of DM)				2
C8/C10 product[4] (g/kg of DM)			450	
Extruded linseed product[5] (g/kg of DM)		1000		
DM (g/kg)	899	909	904	897
GE (MJ/kg of DM)	23.5	22.8	19.5	23.3
Ash (g/kg of DM)	42	52	209	49
CP (g/kg of DM)	238	209	168	232
NDF (g/kg of DM)	232	249	108	282
Crude fat (g/kg of DM)	239	207	241	255

[1] CON2 = control diet; UNSAT = rumen-inert fat source in CON2 was replaced isolipidically by an extruded linseed product; MCFA = rumen-inert fat source in CON2 was replaced isolipidically by a source containing C8:0 and C10:0 fatty acids; DADS2 = diet supplemented with 200 mg/kg DM of diallyl disulfide.
[2] Hyprofat, Provimi B.V., Rotterdam, the Netherlands.
[3] 10% diallyldisufide (Vetcare PVT, Bangalore, India), 90% silica (Provimi France, Treize Vents, France).
[4] 45% fatty acids (50/50 mixture of C8:0/C10:0; Aveve, Leuven, Belgium), 55% silica (Provimi France).
[5] 50% extruded linseed, 2% rapeseed, 18% sunflower meal, 30% wheat bran (Promax 20/20, Provimi France).

The addition of DADS, YP or CAFU did not affect animal performance in Exp. 1 (*Table 3.3*). In Exp. 2, the addition of MCFA significantly increased milk fat concentration, whereas MUN tended to be lower for UNSAT. Other performance parameters were unaffected by the addition of MCFA, UNSAT, or DADS2 in Exp. 2. In comparison with that in Exp. 1, milk production in Exp. 2 was lower for the cows, whereas milk fat and protein concentrations were higher.

Methane Production

Methane production was unaffected by the treatments imposed in these experiments (*Table 3.4*). A considerable difference in the level of methane production was observed between Exp. 1 and Exp. 2, when expressed as the absolute amount (g/d) or per unit of DMI or milk production.

Table 3.3

Dry matter intake, milk production, and milk composition of dairy cows fed diets containing diallyl disulfide, yucca powder, calcium fumarate, a product containing extruded linseed, or a mixture of C8:0/C10:0 fatty acids[1] (n = 10/treatment)

	Experiment 1						Experiment 2					
Item	CON1	DADS1	YP	CAFU	SEM	P-value	CON2	UNSAT	MCFA	DADS2	SEM	P-value
DMI (kg/d)	17.7	17.9	17.4	16.8	0.52	0.819	16.2	16.9	16.7	16.4	0.94	0.952
Milk (kg/d)	30.3	29.6	29.8	28.7	1.18	0.997	24.6	25.3	22.5	24.7	1.03	0.260
Fat (%)	3.97	4.01	3.96	3.95	0.187	0.755	4.82[a]	4.47[a]	5.38[b]	4.52[a]	0.151	<0.001
Protein (%)	3.15	3.24	3.27	3.18	0.089	0.682	3.40	3.34	3.58	3.41	0.077	0.170
Lactose (%)	4.72	4.65	4.73	4.69	0.049	0.250	4.58	4.62	4.58	4.54	0.044	0.603
MUN (mg/dL)	13.6	12.6	12.9	12.4	0.44	0.091	11.6	10.7	11.7	11.6	0.49	0.075
SCC (*1,000 cells/mL)	103	150	153	115	59.3	0.890	315	288	74	288	129.3	0.529

[a,b] Data with different superscripts in the same row within experiment differ significantly (P < 0.05).

[1] CON1 = control diet in Exp. 1; DADS1 = diet supplemented with 56 mg/kg DM of diallyl disulfide; YP = diet supplemented with 3 g/kg of DM yucca powder; CAFU = diet supplemented with 25 g/kg DM of calcium fumarate; CON2 = control diet in Exp. 2; UNSAT = rumen-inert fat source in CON2 was replaced isolipidically by an extruded linseed product; MCFA = rumen-inert fat source in CON2 was replaced isolipidically by a source containing C8:0 and C10:0 fatty acids; DADS2 = diet supplemented with 200 mg/kg DM of diallyl disulfide.

Table 3.4

Methane production of dairy cows fed control diets or diets containing diallyl disulfide, yucca powder, calcium fumarate, a product containing extruded linseed or a mixture of C8:0/C10:0 fatty acids (n = 5 /treatment)[1]

	Experiment 1						Experiment 2					
Item	CON1	DADS1	YP	CAFU	SEM	P-value	CON2	UNSAT	MCFA	DADS2	SEM	P-value
CH_4 (g/cow per day)	450	454	445	424	12.5	0.352	372	393	388	385	25.2	0.941
CH_4 (g/kg of DMI)	25.5	25.4	25.6	25.2	0.40	0.862	22.9	23.2	23.2	23.5	0.49	0.859
CH_4 (g/kg of milk)	15.0	15.4	15.0	14.8	0.67	0.935	15.7	16.1	18.1	15.6	1.77	0.714
CH_4 (% of gross energy intake)	7.3	7.3	7.4	7.4	0.11	0.910	6.3	6.4	6.6	6.5	0.16	0.670

[1] CON1 = control diet in Exp. 1; DADS1 = diet supplemented with 56 mg/kg DM of diallyl disulfide; YP = diet supplemented with 3 g/kg of DM yucca powder; CAFU = diet supplemented with 25 g/kg DM of calcium fumarate; CON2 = control diet in Exp. 2; UNSAT = rumen-inert fat source in CON2 was replaced isolipidically by an extruded linseed product; MCFA = rumen-inert fat source in CON2 was replaced isolipidically by a source containing C8:0 and C10:0 fatty acids; DADS2 = diet supplemented with 200 mg/kg DM of diallyl disulfide.

Table 3.5

Energy balance of dairy cows fed control diets or diets containing diallyl disulfide, yucca powder, calcium fumarate, a product containing extruded linseed, or a mixture of C8:0/C10:0 fatty acids (n = 5 /treatment)[1]

	Experiment 1						Experiment 2					
Item	CON1	DADS1	YP	CAFU	SEM	P-value	CON2	UNSAT	MCFA	DADS2	SEM	P-value
Metabolic weight (kg/cow)	120	121	120	121	2.3	0.988	120	121	122	122	2.8	0.943
Gross energy intake (kJ/kg$^{0.75}$/d)	2839	2857	2782	2647	70.3	0.182	2726	2788	2670	2709	103.1	0.876
Metabolizable energy intake (kJ/kg$^{0.75}$/d)	1704	1701	1661	1578	47.7	0.252	1653	1682	1644	1631	67.0	0.957
ME:GE ratio (%)	60.0	59.5	59.7	59.6	0.40	0.793	60.7	60.4	61.6	60.1	0.65	0.419
Methane production (kJ/kg$^{0.75}$/d)	208	208	206	195	4.6	0.169	171	180	175	175	9.7	0.940
Heat production (kJ/kg$^{0.75}$/d)	1058	1047	1045	1026	19.6	0.712	946	948	975	931	30.8	0.791
Energy in milk (kJ/kg$^{0.75}$/d)	787	762	765	740	25.8	0.662	696	679	668	672	46.4	0.974
Energy retention total (kJ/kg$^{0.75}$/d)	-141	-108	-148	-188	28.6	0.306	11	55	1	29	47.0	0.856
Energy retention protein (kJ/kg$^{0.75}$/d)	-6	-5	-6	-8	0.7	0.100	31	33	37	28	14.0	0.976
Energy retention fat (kJ/kg$^{0.75}$/d)	-135	-103	-143	-181	27.9	0.305	-20	22	-36	0	38.5	0.738
NDF digestibility (%)	69.5	70.1	67.5	64.9	1.72	0.176	69.2	69.2	69.2	69.9	1.61	0.983
CFAT digestibility (%)	59.3	60.8	58.5	53.4	2.18	0.134	59.8[a]	66.7[a]	78.5[b]	60.6[a]	1.36	<0.001

[a,b] Data with different superscripts in the same row within experiment differ significantly (P < 0.05).

[1] CON1 = control diet in Exp. 1; DADS1 = diet supplemented with 56 mg/kg DM of diallyl disulfide; YP = diet supplemented with 3 g/kg of DM yucca powder; CAFU = diet supplemented with 25 g/kg DM of calcium fumarate; CON2 = control diet in Exp. 2; UNSAT = rumen-inert fat source in CON2 was replaced isolipidically by an extruded linseed product; MCFA = rumen-inert fat source in CON2 was replaced isolipidically by a source containing C8:0 and C10:0 fatty acids; DADS2 = diet supplemented with 200 mg/kg DM of diallyl disulfide.

Energy Balance and Digestibility

In Exp.1, energy retention was negative for all treatments and unaffected by treatment (*Table 3.5*). In Exp. 2, energy retention was also unaffected by treatment, but was approximately zero for all treatments. Cows consumed similar amounts of ME in both experiments, but those in Exp. 2 generated less energy as milk, heat and methane. Digestibility of NDF and fat did not differ between treatments in Exp. 1. In Exp. 2, NDF digestibility was unaffected by treatment, but fat digestibility was higher with MCFA than with all other treatments.

Discussion

DADS

To our knowledge, this work is the first evaluation of in vivo effects of dietary DADS on methane emission and animal performance in dairy cows. Garlic oil is known to possess antimicrobial properties and has been shown to decrease methane production in vitro (Chaves et al., 2008, García-González et al., 2008). The main component of garlic oil, DADS, is also known to decrease methane emissions in vitro (Busquet et al., 2005b),

but this has not yet been confirmed in vivo. Diallyl disulfide has been hypothesized to directly inhibit the enzyme 3-hydroxy-2-methyl-glutaryl coenzyme A in human cholesterol synthesis (Gebhardt and Beck, 1996). Archaea have membrane lipids that contain isoprenoid units, the synthesis of which uses the same precursors as human cholesterol synthesis. It has been demonstrated previously that cholesterol-lowering compounds that inhibit 3-hydroxy-2-methyl-glutaryl coenzyme A, lovastatin and mevastatin, can inhibit the growth of rumen methanogens and methane production in vitro (Miller and Wolin, 2001).

In the in vitro study of Busquet et al. (2005b), a significant methane decrease was observed at a concentration of 300 mg of DADS/L in a batch culture system. In the experiment of Kamel et al. (2008), 3 levels of DADS (0.5, 5 and 10 mg of DADS/L) were investigated for their methane-suppressing effect. None of the doses used had a suppressing effect. Apparently, the lowest effective dose of DADS for methane reduction lies in the range of 10 to 300 mg DADS/L, when tested in in vitro batch culture systems.

The level of DADS employed in the study of Busquet et al (2005b) corresponds to a level of 30,000 mg/kg substrate. In the study of Kamel et al (2008), the levels corresponded to 50, 500 and 1,000 mg DADS/kg of substrate, respectively. In our studies, DADS was fed at levels of 56 mg/kg of DM and 200 mg/kg of DM in Exp. 1 and Exp. 2, respectively. This is equivalent to 1.0 or 3.3 g/cow per day respectively. The dose level in Exp. 1 was selected to prevent the occurrence of garlic taint in milk. After completion of the first experiment, in which no milk taint was observed, a higher dose of DADS was selected for Exp. 2. However, a clear and distinctive garlic taint was detected by the technical staff and the authors in the milk from the cows on the DADS2 treatment. The doses used in our experiments were clearly lower than the effective dose employed in the in vitro study of Busquet et al. (2005b) and this may explain the lack of effect on methane emissions in the in vivo studies. However, results from the in vivo experiments demonstrate that the applicability of higher doses of DADS is limited due to the occurrence of garlic taint in milk.

Diallyl disulfide did not affect milk production or composition at the inclusion levels tested in these experiments. When garlic essential oils were included in the diet of lactating dairy cows (5 g/cow per day), total VFA concentrations were increased, but animal performance was not affected (Yang et al., 2007). Diallyl disulfide is one of the main components of garlic oil and might be expected to exert similar results on rumen fermentation. However no effects on milk production were observed in these experiments.

YP

Dose-dependent decreases in methane production have been observed in vitro when yucca saponins were added to the incubation medium (Lila et al., 2003). This decrease may be explained by the symbiotic relationship between methanogens and protozoa in the rumen. Saponins have been shown to have strong detergent properties (Cheeke, 2000) and to reduce the number of rumen protozoa by disrupting their cell membrane. Hegarty (1999) proposed that 37% of rumen methanogenesis originated from methanogens living in a methanogen-protozoan symbiotic relationship and Newbold et al. (1995) demonstrated

that elimination of protozoa diminished methane production by 9 to 25% in vitro. Elimination of protozoa thus has the potential to lower methanogenesis. In the experiment of Lovett et al. (2006), using steers, a significant decrease in protozoa numbers was observed in response to yucca extract (1.2 and 2.6 g/kg of DM, respectively). We used a higher dose (3 g/kg of DM) of YP to obtain this defaunating effect and the consequent reduction in methane production.

However, we observed no effect of YP on methane production. Thus, our findings of a lack of an effect of yucca on methane emissions confirm the findings of Holtshausen et al. (2009), who reported no differences in the number of protozoa when yucca powder was fed at 10 g/kg DM. The effectiveness of different froms of yucca products might differ; in the study of Lovett et al. (2006) yucca extract was used, which is likely to contain a higher concentration of saponins than the yucca powder used in the current study (Cheeke, 2000).

In a meta-analysis, Eugene et al. (2004) concluded that too few data are available in the literature to draw sound conclusions concerning the effects of defaunation on dairy cow performance. In the current experiment, feeding yucca powder did not affect milk production or milk composition, supporting the findings of other researchers (Valdez et al., 1986, Wilson et al., 1998, Lovett et al., 2006, Holtshausen et al., 2009).

The meta-analysis of Eugène et al. (2004) demonstrated that defaunation increased the efficiency of microbial protein synthesis and the flow of microbial protein to the duodenum. Consequently, defaunation would be expected to be especially effective in enhancing animal performance when diets are limiting in MP. In the current experiment, diets were formulated to meet or exceed requirements for MP of the dairy cows, which may explain the lack of response of production parameters.

CAFU

The use of fumarate in methane mitigation has been researched extensively both in vitro (Asanuma et al., 1999, García-Martínez et al., 2005) and in vivo. The results of in vivo experiments have been variable, with some reports of decreased methane production (Bayaru et al., 2001, Wallace et al., 2006) and others reporting no effect (McGinn et al., 2004, Beauchemin and McGinn, 2006, Kolver and Aspin, 2006, McCourt et al., 2008). Methane reductions through fumarate feeding are hypothesized to originate from the consumption of hydrogen in the conversion of fumarate to propionate. However, if the considerable amount of Ca-fumarate (420 g/cow per day) fed in this experiment had been fully converted to propionate, this would have decreased methane emissions by only 11 g/d (2.6%). The actual, nonsignificant decrease in methane production observed in this experiment (-5.8%) was greater than the potential reduction. Moreover, Ungerfeld et al. (2007) demonstrated, by meta-analysis of in vitro data, that fumarate is often not fully converted to propionate, but also to acetate, generating hydrogen. This almost entirely offset the hydrogen used in propionogenesis. The large quantity of fumarate that would be required to achieve substantial reductions in methane production, together with its costs and poor palatability, precludes the use of this substance as a methane inhibitor.

In this experiment, the addition of fumarate to dairy cow diets did not affect milk yield from dairy cows, supporting the findings from previous research (Kolver and Aspin, 2006; McCourt et al., 2008). Milk composition was also unaffected in both other studies, except for the lactose content in the study of Kolver and Aspin (2006), which was higher for the fumarate-fed cows.

Increasing the Unsaturated Fatty Acid Content of the Diet

Dietary unsaturated fat may affect methane production in several ways: indirectly, through decreased DMI or dilution of fermentable OM; through direct toxic effects on the rumen microflora; or by consumption of hydrogen during biohydrogenation (Martin et al., 2010). In Exp. 2 we have ruled out indirect effects of fat addition by providing equal amounts of fat in each treatment and by restricting DMI. In this way, any effects on methane emissions could only have come from a direct effect of the increased dietary content of unsaturated fatty acids on the rumen microflora or by the hydrogen sink function of the unsaturated fatty acids supplied by the product containing the extruded linseed. We hypothesized that the increased content of dietary unsaturated fatty acids would lower methanogenesis due to specific effects of these fatty acids on methanogenesis observed in earlier research (Czerkawski et al., 1966b, Prins et al., 1972).

Products containing extruded linseed, a source rich in C18:2 and C18:3, have been demonstrated to reduce methane production when added to dairy cow rations (Martin et al., 2008), but this reduction appeared to originate mainly from a reduction in DMI and NDF digestibility: methane production expressed per unit of digested NDF was unaffected. In the current experiment, no methane-lowering effect was observed when fractionated palm oil was isolipidically exchanged for a product containing extruded linseed. In this experiment, apparent total tract digestibility of NDF was unaffected by supplementation with the product containing extruded linseed. The methane-suppressing effects of C18:2 and C18:3 observed in earlier research may be due to a more general toxic effect on the rumen microbes, rather than a specific toxic effect on the rumen methanogens alone (Maia et al., 2007).

In our experiment, cows consumed approximately 850 g of DM/d of the extruded linseed product, which contained 20.7% crude fat (352 g linseed oil/d). Linseed oil consists mainly of C18:2 and C18:3 fatty acids and if all the double bonds in this molecule would be hydrogenated in the rumen, this would reduce methane emissions by approximately 6 g/d or 1.6% (Martin et al., 2010).

It is thus likely that our approach would reveal the direct effect of unsaturated fatty acids on the rumen microflora and consequently methane production. The fact that no differences in methane production were observed may mean that the mechanism of methane reduction by products containing extruded linseed is due mainly to indirect effects (e.g. reduced NDF digestibility, reduced DMI, dilution of fermentable OM) rather than a direct toxic effect on the rumen methanogens. Eugene et al. (2008) concluded that the methane reduction observed as a consequence of fat or oil consumption was mainly due to a reduction in DMI, which may originate from a reduced NDF digestibility.

Fat-rich feed materials such as extruded linseeds can be utilized to enhance the dietary energy content of dairy cow diets and stimulate milk production. Indeed, enhancing dietary energy content by including linseed oil increased milk production (Bu et al., 2007). However, in the experiment of Martin et al. (2008), the addition of extruded linseed significantly lowered DMI and lowered milk production despite an increase in dietary energy content. The inclusion of extruded linseed lowered rumen digestibility of OM and in particular NDF in that experiment. It is generally recommended not to exceed crude fat levels of 6.5% DM (NRC, 2001). The addition of extruded linseed to the diet did not affect dairy cow performance in our study. Feeding the extruded linseed product tended to decrease MUN contents; this may have been a consequence of the lower CP content of the mixture containing the extruded linseed.

Capric and Caprylic Acid

Caprylic acid and capric acid were demonstrated to lower methanogenesis in vitro (Ajisaka et al., 2002). These authors added these fatty acids to 2 different matrices (α-cyclodextrin or β-cyclodextrin) to produce a solid feed material, similar to the procedure followed in the current in vivo experiment. A reduction of 60% in methane reduction was observed when 40 mg of capric acid on the β-cyclodextrin carrier was added to 60 mL medium (0.7 g/L or 139 g/kg substrate) and a nonsignificant 40% reduction in methane production was observed when 20 mg of capric acid was added. This observation was later confirmed for capric acid by Goel et al. (2009) who found methane reductions of 45 and 88%, respectively, when 20 or 30 mg of capric acid were added to 50 mL incubation medium with 0.5 g substrate (40 or 60 g/kg substrate, respectively). Dohme et al. (2001) observed no reduction in methane production when C8:0 or C10:0 were added to a Rusitec system at 0.6 g/L or 50 g/kg DM substrate.

In the current experiment, which is the first to investigate the in vivo effects of these fatty acids on methanogenesis, cows on the MCFA treatment consumed 16.7 kg DM containing 45 g of product/kg DM. This product contained 45% fatty acids, so the amount of C8:0 or C10:0 consumed was 169 g/cow per day or 10 g/kg of DM of each fatty acid. However, when concentrations of C8:0 or C10:0 are expressed in relation to the substrate supplied to the in vitro system (40 to 139 g fatty acids/kg substrate), concentrations provided in the in vitro systems were higher than those in the in vivo experiment.

The addition of C8:0 and C10:0 increased milk fat content, but did not affect milk yield or milk protein content. Fat digestibility was higher on the MCFA treatment than for the other treatments, providing a possible explanation for the higher milk fat contents.

Difference in Methane Production Between Experiments

A considerable difference in the overall level of methane production was observed between experiments (443 g/d for Exp. 1 and 385 g/d for Exp. 2), although the dietary composition was broadly similar in both experiments. The crude fat content of the TMR used in Exp. 2 was clearly higher than for Exp. 1 (58 g/kg of DM vs. 33 g/kg of DM for Exp. 2 and Exp. 1, respectively). Eugene et al. (2008) conducted a meta-analysis and provided an equation to predict methane emission from DMI and daily lipid intake.

Using of this equation results in predicted methane productions of 328 g/d and 299 g/d for Exp. 1 and Exp. 2, respectively. Although the absolute level of methane production observed in both experiments was higher than predicted, the difference in methane production predicted from the model is similar to the observed difference in our experiments (29 g/d for the prediction equation vs. 22 g/d observed between Exp. 1 and 2), providing a likely explanation for the difference in methane emission between experiments.

The test products had no effect on methane production in either experiment, whereas their efficacy had been demonstrated in vitro. These findings emphasize that results observed in vitro should be confirmed in vivo (Flachowsky and Lebzien, 2009a). It also shows that in vitro experiments showing significant methane reductions often use concentrations of the active ingredient, expressed in grams per kilogram of substrate, that are not practical to use in vivo.

Conclusions

Addition of diallyl disulfide, yucca powder, calcium fumarate, a product containing extruded linseed, or a mixture of capric and caprylic acid to dairy cow diets did not affect enteric methane emissions or energy balance in concentrations that have practical applications. Fat digestibility and milk fat content were elevated by the addition of caprylic and capric acid to the diet.

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CHAPTER 4

Nitrate and sulfate: Effective alternative hydrogen sinks for mitigation of ruminal methane production in sheep

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ABSTRACT

Twenty male crossbred Texel lambs were used in a 2 x 2 factorial design experiment to assess the effect of dietary addition of nitrate (2.6% of dry matter) and sulfate (2.6% of dry matter) on enteric methane emissions, rumen volatile fatty acid concentrations, rumen microbial composition, and the occurrence of methemoglobinemia. Lambs were gradually introduced to nitrate and sulfate in a corn silage-based diet over a period of 4 wk, and methane production was subsequently determined in respiration chambers. Diets were given at 95% of the lowest ad libitum intake observed within one block in the week before methane yield was measured to ensure equal feed intake of animals between treatments. All diets were formulated to be iso-nitrogenous. Methane production decreased with both supplements (nitrate: -32%, sulfate: -16% and nitrate + sulfate: -47% relative to control). The decrease in methane production due to nitrate feeding was most pronounced in the period immediately after feeding, whereas the decrease in methane yield due to sulfate feeding was observed during the entire day. Methane-suppressing effects of nitrate and sulfate were independent and additive. The highest methemoglobin value observed in the blood of the nitrate-fed animals was 7% of hemoglobin. When nitrate was fed in combination with sulfate, methemoglobin remained below the detection limit of 2% of hemoglobin. Dietary nitrate reduced heat production (-7%), whereas supplementation with sulfate increased heat production (+3%). Feeding nitrate or sulfate had no effects on volatile fatty acid concentrations in rumen fluid samples taken 24 h after feeding, except for the molar proportion of branched-chain volatile fatty acids, which was higher when sulfate was fed and lower when nitrate was fed, but not different when both products were included in the diet. The total number of rumen bacteria increased as a result of sulfate inclusion in the diet. The number of methanogens was reduced when nitrate was fed. Enhanced levels of sulfate in the diet increased the number of sulfate-reducing bacteria. The number of protozoa was not affected by nitrate or sulfate addition. Supplementation of a diet with nitrate and sulfate is an effective means for mitigating enteric methane emissions from sheep.

INTRODUCTION

Rumen fermentation results in the production of excess hydrogen, which needs to be removed from the rumen for the fermentation process and microbial growth to continue efficiently (Immig, 1996). In general, hydrogen is removed through the activity of methanogenic Archaea, which reduce carbon dioxide with hydrogen to generate methane and water. For most feeds consumed by ruminants, methanogenesis is the main route of hydrogen disposal during anaerobic rumen fermentation (Beauchemin et al., 2008). The methane resulting from methanogenesis represents a loss of dietary energy to the animal (Johnson and Johnson, 1995) and it is a significant greenhouse gas (Steinfeld et al., 2006). These factors have led to a global search for nutritional strategies to mitigate methane emission from ruminants.

One strategy is to redirect hydrogen into processes that yield beneficial products for the ruminant. Examples include the stimulation of propionogenesis by addition of substrates to the diet that support propionate production and attempts to introduce bacteria expressing reductive acetogenesis into the rumen (Joblin, 1999, Molano et al., 2008). These processes would yield propionate or acetate, respectively, as nutrients for the animal and at the same time would lower the hydrogen availability for methanogenesis. However, the introduction of propionate precursors (i.e. malate and fumarate) in ruminant diets has resulted in variable effects on methane production (Beauchemin and McGinn, 2006, Wallace et al., 2006, Foley et al., 2009) and attempts to establish acetogenic bacteria in the rumen have failed because of a lower affinity of the acetogenic bacteria for H_2 when compared with methanogenic Archaea (Le Van et al., 1998, Ellis et al., 2008).

The possibility of nitrate as an alternative hydrogen sink to carbon dioxide has been downplayed because of the possible toxic effects of nitrite, which is formed as an intermediate during the reduction of nitrate to ammonia (Lewis, 1951). A few reports have examined the potential of nitrate as methane-lowering feed additive, and it appears to lower methanogenesis consistently. However, lowered methane yields were found in vitro (Allison and Reddy, 1984, Guo et al., 2009) and in vivo in animals where the nitrate was dosed once daily directly into the rumen (Sar et al., 2004, Takahashi et al., 1998). Studies of effects of feeding a nitrate source on methane production appear to be lacking.

The reduction of nitrate to nitrite (Gibbs free energy, ΔG_0 = -130 kJ/mol of hydrogen; Ungerfeld and Kohn (2006)) and the subsequent reduction of nitrite to ammonia (ΔG_0 = -124 kJ/mol of hydrogen ; Ungerfeld and Kohn (2006)) yield more energy than the reduction of carbon dioxide to methane (ΔG_0 = -16.9 kJ/mol of hydrogen ; Ungerfeld and Kohn (2006)). These processes could be the principal route of hydrogen disposal if sufficient nitrate is available in an actively fermenting rumen ecosystem. The reduction of nitrate to ammonia consumes 8 electrons and each mole of nitrate reduced could thus lower methane production by 1 mole. The ammonia generated will be available for anabolism and would be an important supply of fermentable N on diets deficient in CP where low rumen ammonia may limit microbial protein synthesis (Leng and Nolan, 1984, Dijkstra et al., 1998).

In animals unadapted to nitrate in their diet, the capacity of the rumen microflora to reduce nitrate to nitrite exceeds the capacity for nitrite reduction (Lewis, 1951). This leads to accumulation of nitrite in the rumen, which is readily absorbed across the rumen wall and converts blood hemoglobin (Hb) from the ferrous (Fe^{2+}) to the ferric (Fe^{3+}) form. The ferric form of hemoglobin, methemoglobin (MetHb), renders the molecule incapable of transporting oxygen to the tissues (Morris et al., 1958). The resulting condition, methemoglobinemia, is a state of general anoxia, which in mild cases may depress animal performance, but in severe cases may be fatal (Ozmen et al., 2005).

Careful stepwise introduction of nitrate in the diet of sheep allows the rumen microflora to adapt and increase their capacity to reduce both nitrate and nitrite (Alaboudi and Jones, 1985). Sheep gradually adapted over a period of 10 wk to high nitrate diets (1.5 g of nitrate/kg of BW per day) exhibited no clinical signs of methemoglobinemia (Alaboudi and Jones, 1985). In some anaerobic environments, hydrogen sulfide appears to play a role as electron donor in the reduction of nitrite to ammonia by nitrate-reducing sulfide-oxidizing bacteria (Hubert and Voordouw, 2007). Supplementation of the diet with sulfur (Leng, 2008) or cystein (Takahashi et al., 1998) may therefore reduce nitrite accumulation in the rumen. Sulfate is a reductant (ΔG_0 = -21.1 kJ/mol of hydrogen; Ungerfeld and Kohn, 2006) and will also compete for electrons and may lower methane production.

In the current experiment the methane-lowering potential of dietary nitrate and sulfate was evaluated after gradual introduction of these compounds to the maximum levels over a 4-wk adaptation period. Our hypothesis is that addition of nitrate to a diet for sheep would reduce methane emissions from enteric fermentation and that an additional sulfate source would prevent methemoglobinemia and provide an additional decrease of methane emission.

MATERIALS AND METHODS

Experimental Design

The experimental design was a 2 x 2 factorial, with dietary nitrate and sulfate concentration as the independent factors. At the start of the experiment, 20 sheep were blocked by weight (5 blocks of 4 sheep) and allocated randomly within a block to 1 of the 4 dietary treatments. Treatments consisted of a control treatment (no addition of nitrate or sulfate), a nitrate treatment (inclusion of 2.6% nitrate in dietary DM), a sulfate treatment (inclusion of 2.6% sulfate in dietary DM) and a treatment including both molecules in the diet (2.6% nitrate and 2.6% sulfate).

Animals and Housing

The experiment was approved by the Institutional Animal Care and Use Committee of the Animal Sciences Group, WUR, Lelystad, the Netherlands. The experiment was conducted with 20 male crossbred Texel lambs, with initial BW of 42.9 ± 4.3 kg (mean ± SD).

During a 4-wk period of adaptation to the dietary additives, animals were housed in individual calf hutches (195 x 150 x 145 cm) to allow individual feeding. This adaptation period served to gradually acclimate the animals to the levels of nitrate and sulfate in their diet to allow the rumen microflora and fauna to adapt. During the adaptation period, formaldehyde-treated soybean meal was replaced by the experimental concentrates in weekly increments of 25%. Sheep were weighed weekly during the entire experiment.

Following the adaptation period, 4 animals (one block) were housed individually in respiration chambers for 1 wk to determine gaseous exchanges. A new block of sheep was introduced to the chambers each week. Sheep remained housed in the calf hutches until they were transported to the respiration chambers. The first block had been fed additional nitrate and sulfate for 4 wk (the adaptation phase), whereas the last block had received the dietary additives for 8 wk before measurement of gaseous exchange. The data on the adaptation period involve the first 4 wk of the experiment, whereas the data on the experimental period involve the periods in the respiration chambers.

The indirect calorimetry respiration chambers used were described in detail by Verstegen et al. (1987). Temperature was maintained at 15°C, and relative humidity at 70%. Two types of chambers (length x width x height: 100 x 80 x 97 cm, 2 chambers, and 100 x 150 x 200 cm, 2 chambers, respectively) were used. Sheep were allocated to the type of chamber in such a way that each treatment was repeated 2 or 3 times within one type of chamber. Ventilation rate was 70 L/min for the smaller type of chambers and 90 L/min for the other type. Consumption of oxygen and production of carbon dioxide and methane were determined per chamber in 9-min intervals as described by Verstegen et al. (1987).

Feeding

The complete ration consisted of a basal diet and a concentrate in meal form that contained the dietary additives (90 and 10% respectively, DM basis; *Table 4.1*). The concentrate and basal diet were hand-mixed daily before feeding. Water was freely available during the experiment. Samples (±500 g) of the concentrates and roughages fed were collected on d 4 of each measurement week in the respiration chambers. After the experiment, samples were stored frozen at -20°C pending further analyses.

Table 4.1

Feed ingredient composition of experimental rations (g/kg of DM) fed to growing male lambs

	$-NO_3$		$+NO_3$	
	$-SO_4$	$+SO_4$	$-SO_4$	$+SO_4$
Item				
Basal diet[1]	90.0	90.0	90.0	90.0
Concentrate				
Formaldehyde-treated soybean meal	2.2	2.2	2.3	2.3
Urea	1.5	1.5		
Nitrate source[2]			3.4	3.4
$MgSO_4$ (anhydrous)		3.3		3.5
MgO	1.3		1.4	
Limestone	2.2	2.2		
Wood cellulose	2.8	0.8	2.9	0.8

[1] Containing maize silage, 74%; chopped barley straw, 16%; formaldehyde treated soybean meal, 9%; and a mineral premix, 1% on a DM-basis
[2] $5Ca(NO_3)_2 \cdot NH_4NO_3 \cdot 10H_2O$; 75% NO_3 in DM

Lambs were given their ration once daily at 0800 h. Before each morning feeding, orts were removed from the feed bins and weighed to determine feed intake. During the adaptation period, feed was available ad libitum. During the measurement week in the respiration chambers, feed availability was restricted to allow comparison of the effects of dietary treatments on methane production without the potential confounding effect of feed intake differences. Feed availability was restricted to 95% of the feed consumed by the animal consuming the least feed within a block in the week before housing animals in the respiration chambers.

Control and nitrate containing diets were designed to be isonitrogenous by the substitution of nitrate for urea. Limestone, MgO, and wood cellulose were used to ensure equal Ca, Mg and DM concentrations among mixtures.

Analyses in Feed

Feed samples were thawed and ground over a 1-mm screen before analysis. Dry matter content was determined by drying for 16 h at 70°C in a forced-air oven. Nitrogen content was determined according to the Dumas method (ISO 16634-1; ISO, 2008). Crude protein content was calculated by multiplying total N content by 6.25. Crude fat content was determined by ether extraction according to (ISO 6492; ISO, 1999). Sugar content was determined by ethanol (40% vol/vol) extraction and subsequent titrimetric determination of reducing sugars (as glucose) according to the Luff-Schoorl method (NEN 3571; NEN, 1974). The method of Ewers (ISO 6493: ISO, 2000) was used for determination of starch content. Neutral detergent fiber was determined according to Van Soest et al. (1991) after

pretreatment with amylase. The NDF contents reported include residual ash. For nitrate and sulfate analysis, 2.5-g samples were extracted for 30 min in 200 mL of distilled water. The solution was filtered twice (0.45-µm filter paper) and 5mL of the filtrate was used for determination of nitrate and sulfate contents by ionchromatography (DX120 ionchromatograph, Dionex, Sunnyvale, CA).

Blood and Rumen Fluid Sampling

Blood was sampled during the adaptation period at d 2, 8, 15, 22 and 28 at 1, 3 and 5 h postfeeding. Days 2, 8, 15 and 22 were 1 d after the nitrate or sulfate was incrementally (25%) increased in the diet. On d 28, lambs had been on 100% of the dietary treatments for 1 wk. Blood samples were taken from the jugular vein in heparinized collection tubes (Vacutainers, Becton Dickinson, Breda, the Netherlands) and stored at 4°C immediately after sampling. At the end of the sampling day, samples were dispatched for analyses and were analyzed the next day. The MetHb content of the blood was determined according to the methods described in Evelyn and Malloy (1938).

After completion of the sampling period in the respiration chambers, each block of sheep was transported to a slaughterhouse (20 km) and rumen fluid samples (200 mL) were taken within 1 h after slaughter (approximately 0900 h; some 24 h following their last feed). Rumen contents were squeezed manually to obtain the rumen fluid samples. The rumen fluid samples also contained small particles. Flasks containing rumen fluid samples were immediately immersed in a bucket of ice water to stop microbial fermentation and, once all samples had been taken, stored at –20°C until analysis.

Determination of VFA Concentration and Microbial Composition

Rumen fluid samples were thawed and centrifuged at 3,000 x *g* for 10 min. Volatile fatty acids [acetate, propionate, butyrate, valerate and branched chain fatty acids (BCVFA)], were analyzed from the supernatant by GC using pivalic acid as an internal standard method as described previously (Holben et al., 2002). The group of BCVFA included isobutyric acid, 2-methyl-butyric acid, and isovaleric acid.

Microbes in the rumen fluid subsamples were analyzed for eubacteria, methanogens, sulfate-reducing bacteria, and protozoa. For this analysis, samples were subjected to quantitative bacterial lysis and DNA purification as described for chicken cecal samples (Apajalahti et al., 1998). Quantitative real-time PCR (qPCR) and the primers specific to eubacteria, methanogens, sulfate-reducing bacteria and protozoa were used for the quantitative analysis as described previously (Nadkarni et al., 2002, Sylvester et al., 2004, Cadillo-Quiroz et al., 2006).

Calculations and Statistical Analysis

Heat production rates were calculated from gaseous exchange (Brouwer, 1965). Gas exchange and feed intake data, averaged over the last 4 complete 24-h periods of each period were included in the statistical analyses.
Feed intake, daily gas exchange data, bacterial and protozoal numbers in rumen fluid,

and VFA concentrations were analyzed using the MIXED procedure in SAS (2003; SAS Institute Inc., Cary, NC), with intake of nitrate and sulfate and their interaction as well as block included as fixed effects. Chamber type was included as a random effect. Homogeneity of variance of studentized model residuals was checked before the statistical analysis. Data on bacterial and protozoal numbers in rumen fluid were log-transformed before statistical analysis. For all analyses, significance was declared at P = 0.05, and a trend was declared at P = 0.10.

Hourly gas exchange rates, (Figures 4.1 and 4.2: expressed per $kg^{0.75}$ per day) were analyzed by repeated measures ANOVA, using the MIXED procedure in SAS. Intake of nitrate and sulfate and their interaction, as well as block were included as fixed effects. Effects of time and interactions between the fixed treatment effects and time were included taking hourly data obtained from the same sheep as repeated measures, applying first-order autoregressive procedures.

RESULTS

Feed Composition

The nitrate and sulfate concentrations in the experimental concentrates were established as formulated (*Table 4.2*).

In practice, minor differences existed in CP content of the concentrates, but these were only included at 10% of dietary DM, resulting in small differences in the CP content of the total diets. The maximum difference in CP content of the total diet was 7 g of CP/kg DM. The inclusion of wood cellulose led to a marked increase of NDF in concentrates without supplements and with added nitrate only.

Table 4.2

Analyzed chemical composition (g/kg of DM, unless stated otherwise) of feed ingredients and calculated chemical composition of experimental diets[1]

	DM (g/kg)	CP	Starch	Sugar	Crude fat	NDF	NO_3	SO_4
Maize silage	328	74	367	12	40	365	0	0
Formaldehyde-treated soybean meal	889	514	48	133	36	94	1	3
Straw	932	38	15	NA[2]	18	838	1	0
Concentrate CON	942	551	5	30	20	214	0	1
Concentrate NO_3	894	503	6	28	26	229	257	1
Concentrate SO_4	968	494	5	30	27	89	1	262
Concentrate NO_3 + SO_4	918	479	12	33	32	72	247	255
Calculated composition[3]								
Diet CON	416	151	252	22	34	391	0	0
Diet NO_3	414	146	252	21	35	392	26	0
Diet SO_4	417	145	252	22	35	378	0	26
Diet NO_3 + SO_4	415	144	253	22	35	377	25	26

[1] Treatments consisted of a control treatment (CON, no addition of nitrate or sulfate), a nitrate treatment (NO_3, inclusion of 2.6% nitrate in dietary DM), a sulfate treatment (SO_4, inclusion of 2.6% sulfate in dietary DM), and a treatment including both molecules in the diet (NO_3 + SO_4, 2.6% nitrate and 2.6% sulfate).
[2] Not analyzed.
[3] Calculated chemical composition of diets based on analyzed chemical composition of feed ingredients.

Methemoglobin in Blood During the Adaptation Period

During supplementation of the diet with 25% or 50% of the final concentrate inclusion rate, none of the sheep had detectable blood MetHb concentrations (< 2% of Hb). At 75% of the final inclusion rate, 1 sheep on the nitrate diet tested positive (> 2% of Hb) at 3 h postfeeding, but the MetHb value was only 3% of Hb. At the 100% inclusion rate, 2 sheep on the nitrate treatment tested positive with MetHb values of 7 and 3% of Hb respectively, at 3 h postfeeding after having been fed 26 g nitrate/kg of DM for 1 wk (d 28). Blood samples of sheep on the control diet and both sulfate-containing diets were below detectable levels of MetHb.

Feed Intake and Body Weight Gain During the Adaptation to Dietary Nitrate and Sulfate

During the 4-wk adaptation period, no difference in average ad libitum feed intake (average 1.1 kg DM/d) was observed between any of the treatments. Body weight gain (average 2.75 kg in the adaptation period) was also unaffected by treatment.

Effects of Nitrate and Sulfate on Gaseous Exchange

The restricted feeding regimen applied during the period in the respiration chambers resulted in very similar feed intake in all treatments (*Table 4.3*).

Table 4.3

Dry matter intake, gaseous exchange, and heat production of growing male lambs fed nitrate and sulfate sources[1]

Item	$-NO_3$		$+NO_3$		Pooled	P-value of effects		
	$-SO_4$	$+SO_4$	$-SO_4$	$+SO_4$	SEM	NO_3	SO_4	$NO_3 \times SO_4$
DMI (g/d)	999	982	985	990	12.3	0.791	0.647	0.372
CH_4 (L/d)	25.5	21.6	17.3	13.6	1.54	<0.001	0.033	0.941
CH_4 (L/kg of $BW^{0.75}$ per day)	1.48	1.30	1.01	0.80	0.10	<0.001	0.082	0.899
CH_4 (L/kg of $BW^{0.75}$ per day)	25.5	22.0	17.6	13.9	1.54	<0.001	0.041	0.910
CO_2 (L/kg of $BW^{0.75}$ per day)	25.9	26.4	24.3	25.5	0.40	0.011	0.050	0.391
O_2 (L/kg of $BW^{0.75}$ per day)	26.2	27.0	24.3	25.7	0.50	0.008	0.057	0.568
Heat (kJ/kg of $BW^{0.75}$ per day)	550	566	513	542	9.95	0.010	0.048	0.522

[1] NO_3 = nitrate added to the diet (26 g of nitrate/kg DM); SO_4 = sulfate added to the diet (26 g of sulfate/kg DM); + or – indicate whether the component was added or not to the respective treatment.

Dietary nitrate and sulfate supplementation reduced methane production (L/d) by 32% ($P < 0.001$) and 16 % ($P = 0.033$) relative to the control treatment, respectively. No interaction was observed, indicating additivity of these effects. Nitrate addition reduced oxygen consumption by 7% ($P = 0.008$) and carbon dioxide production by 6% ($P = 0.011$), resulting in a reduction of the calculated heat production by 7% ($P = 0.010$) relative to the control treatment.

The addition of sulfate to the diet tended to increase oxygen consumption (+3%; $P = 0.057$) and carbon dioxide production (+3%; $P = 0.050$), resulting in a higher calculated heat production (+3%; $P = 0.048$) for the sulfate-fed sheep relative to the control treatment. No interaction of nitrate and sulfate intake on heat production was observed and thus heat production was not affected by the nitrate + sulfate treatment.

Diurnal Pattern of Methane Production

The diurnal pattern of enteric methane production is presented in *Figure 4.1*.

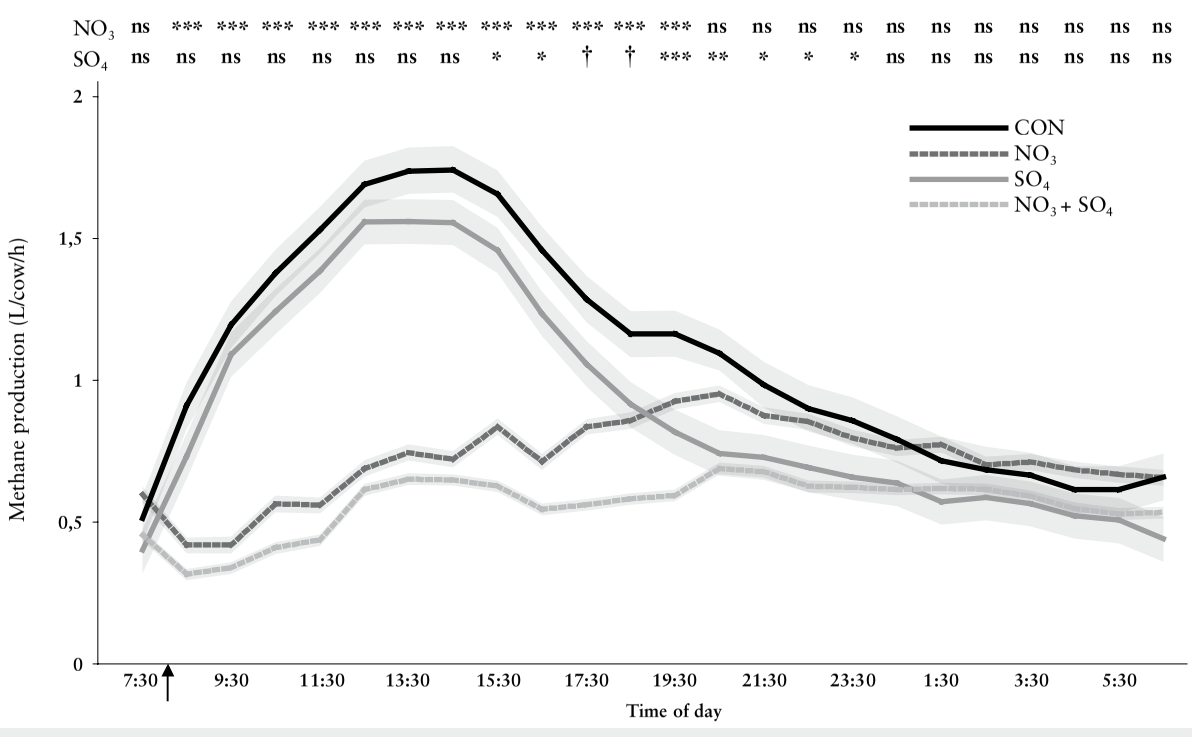

Figure 4.1

Diurnal methane production pattern (L/cow/h) of male lambs fed nitrate and sulfate.

Symbols indicate significance of main effects of NO_3 or SO_4 and their interaction (†P < 0.10; *P < 0.05; **P < 0.01; ***P < 0.001). Arrow indicates time of feeding. Treatments consisted of a control treatment (CON, no addition of nitrate or sulfate), a nitrate treatment (NO_3, inclusion of 2.6% nitrate in dietary DM), a sulfate treatment (SO_4, inclusion of 2.6% sulfate in dietary DM), and a treatment including both molecules in the diet (NO_3 + SO_4, 2.6% nitrate and 2.6% sulfate).

Animals were fed at 0800 h, after which methane production from animals on the control treatment progressively increased to reach a maximum at 5 to 6 h after feeding followed by a gradual decline. The addition of nitrate to the ration invoked a markedly different methane production pattern. Immediately after feeding, methane production rate remained at a much lower level. Twelve hours after feeding, methane production rates returned to a level similar to that of the control animals.
The methane-suppressing effect of sulfate occurred during a different period within the 24-h timeframe than that of nitrate. The largest reduction in methane production became recognizable at 10 h postfeeding. Interactions between nitrate and sulfate intake on methane production were not significant at any point within the 24-h period.

Diurnal Pattern of Heat Production

Heat production was lowered by nitrate feeding, mostly in the period directly after feeding (*Figure 4.2*), coinciding with the period in which methane production was reduced in this treatment.

Sulfate feeding increased heat production, mostly in the period directly after feeding. This resulted in equal rates of heat production between the combined nitrate and sulfate treatment and the treatment without nitrate and sulfate supplement.

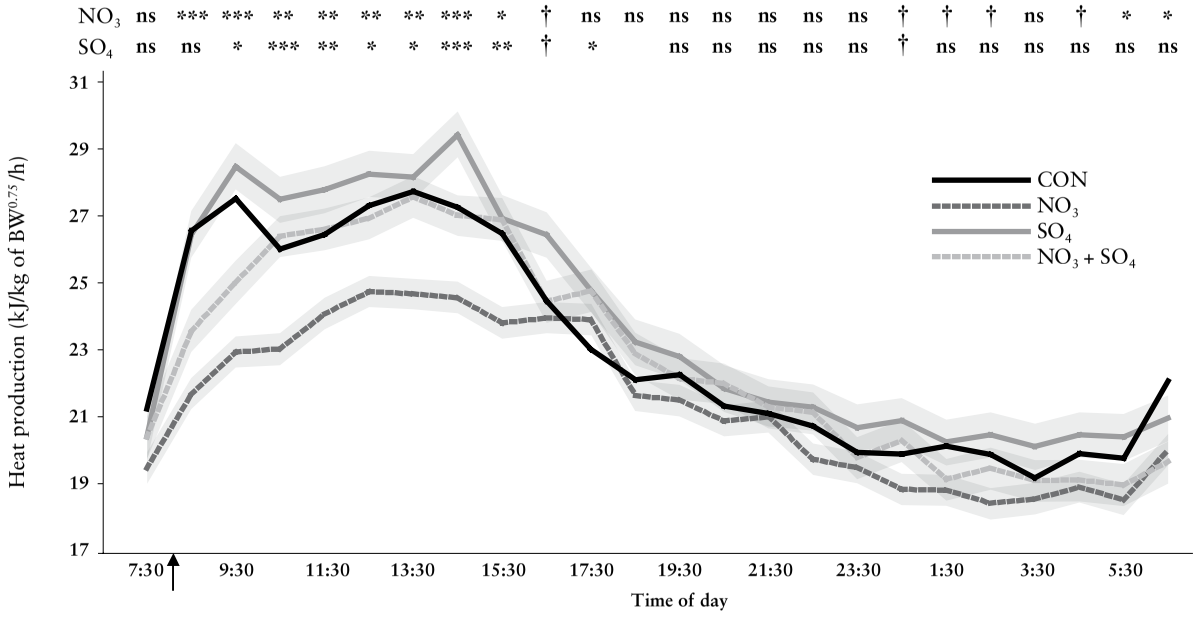

Figure 4.2

Diurnal pattern of heat production (kJ/kg of $BW^{0.75}$/h) of male lambs fed nitrate and sulfate.

Symbols indicate significance of main effects of NO_3 or SO_4 and their interaction (†P < 0.10; *P < 0.05; **P < 0.01; ***P < 0.001). Arrow indicates time of feeding. Treatments consisted of a control treatment (CON, no addition of nitrate or sulfate), a nitrate treatment (NO_3, inclusion of 2.6% nitrate in dietary DM), a sulfate treatment (SO_4, inclusion of 2.6% sulfate in dietary DM), and a treatment including both molecules in the diet (NO_3 + SO_4, 2.6% nitrate and 2.6% sulfate).

Ruminal VFA Concentration and Microbial Composition

Concentrations of VFA in ruminal fluid of lambs on all treatments were not influenced by feeding nitrate or sulfate (*Table 4.4*). Proportions of BCVFA were reduced because of nitrate feeding (P = 0.036), but increased with sulfate feeding (P = 0.015).

Table 4.4

Total VFA concentrations, VFA molar proportions, and microbial composition (log/mL of rumen contents) in rumen contents of growing male lambs fed nitrate and sulfate sources[1]

Item	- NO_3		+ NO_3		Pooled	P-value of effects		
	- SO_4	+ SO_4	- SO_4	+ SO_4	SEM	NO_3	SO_4	NO_3 x SO_4
Total VFA (mM)	47.5	53.2	59.0	52.0	5.90	0.402	0.919	0.300
Acetate (mol/100 mol)	65.6	64.6	65.1	64.5	0.98	0.712	0.442	0.859
Propionate (mol/100 mol)	19.4	20.4	20.9	21.4	0.96	0.226	0.445	0.756
Butyrate (mol/100 mol)	10.0	9.7	10.4	9.1	0.75	0.950	0.315	0.502
BCVFA (mol/100 mol)	3.8	4.1	2.5	3.9	0.31	0.036	0.015	0.117
Valerate (mol/100 mol)	1.3	1.1	1.1	1.2	0.08	0.372	0.433	0.154
NH_4^+ (mM)	9.0	7.8	6.3	7.9	1.91	0.524	0.945	0.473
Total bacteria	10.9	11.1	10.9	11.3	0.16	0.365	0.039	0.829
Total methanogens	9.0	9.1	8.3	8.7	0.12	<0.001	0.071	0.283
Protozoa	6.1	5.9	6.0	5.8	0.15	0.379	0.247	0.790
SO_4 reducers	5.8	7.9	5.0	8.1	0.28	0.313	<0.001	0.084

[1] NO_3 = nitrate added to the diet (26 g of nitrate/kg DM); SO_4 = sulfate added to the diet (26 g of sulfate/kg DM); + or – indicate whether the component was added or not to the respective treatment.

Supplementation with sulfate increased the total number of rumen bacteria in rumen fluid (P = 0.039). The number of methanogens decreased when nitrate was included in the diet (P < 0.001), whereas addition of sulfate tended to increase their numbers (P = 0.071). The protozoa population was unaffected by inclusion of nitrate and sulfate in the feed. Sulfate supplementation significantly increased the number of sulfate-reducing bacteria in the rumen (P < 0.001). The increase in sulfate-reducing bacteria tended to be larger when sulfate was added to nitrate containing diets (trend for a nitrate x sulfate interaction; P = 0.084).

DISCUSSION

Dietary Adaptation and Occurrence of Methemoglobinemia

High doses of nitrate in ruminant diets have been reported to cause methemoglobinemia, decreasing the capacity of blood to transport oxygen to an animals' tissues (Bradley et al., 1939, Lewis, 1951). Clinical toxicity signs are known to occur at MetHb levels of 30 to 40% of Hb and higher (Bruning-Fann and Kaneene, 1993). In this experiment, blood was sampled regularly, and only slightly elevated MetHb levels were found in two sheep (maximum level was 7% of Hb for 1 sheep). Sar et al. (2004) observed MetHb levels of 18.4% of Hb when 0.9 g of nitrate/kg of $BW^{0.75}$ per day was administered intraruminally to sheep in a single load. In another study, Takahashi et al. (1998) pulse-dosed $NaNO_3$ into the rumen of sheep at a rate of 1.1 g of nitrate/kg of $BW^{0.75}$ per day and observed MetHb levels of over 30% of Hb. Although considerably more nitrate (1.6 g of nitrate/kg of $BW^{0.75}$ per day) was provided in our study, lower levels of methemoglobin were observed. The difference in observed MetHb values between our study and the experiments of Sar et al. (2004) and Takahashi et al. (1998) may originate in the method of application to the animal; in our study the nitrate was fed, whereas in the other studies the nitrate was pulse-dosed into the rumen. This pulse-dosing would presumably cause much higher peak values of nitrite in rumen fluid, providing a possible explanation for the higher MetHb values observed in the other studies. Another possible explanation could be that in both other studies sheep were not adapted to nitrate and the rumen microflora possibly had a lower capacity to reduce nitrite than rumen microflora of the sheep in our experiment.

Allison and Reddy (1984) demonstrated that nitrite reduction rates can be increased from 25 nmol/min per mL rumen fluid to 62 nmol/min per mL by feeding nitrate (0.47 g of nitrate/kg of BW per day) to sheep, whereas nitrate reduction rates were increased 26-fold (from 4.5 to 117 nmol/min per mL) compared with sheep fed no additional nitrate in their diets. A similar finding was reported by Alaboudi and Jones (1985) when sheep were slowly introduced to high nitrate diets (1.5 g of nitrate/kg of BW per day). Nitrate concentrations higher than 0.5% of DM in forages can be lethal for unadapted ruminants (Bruning-Fann and Kaneene, 1993), but adaptation apparently enabled rumen bacteria to increase in numbers or increase their nitrite-reducing capacity. Methemoglobin was not detected (detection limit 2% MetHb) in animals on the control treatment and treatments receiving additional sulfate. This indicates that sulfate may play a role in the acceleration of nitrite reduction in the rumen or remove a metabolic restriction to the production of ammonia from nitrite when diets contain high levels of nitrate. The time elapsed between sampling of the blood and the actual analysis for methemoglobin was approximately 24 h. Fukui et al. (1980) reported that 36.1% of the original amount of MetHb was recovered after 24 h storage in a refrigerator and Sleight and Sinha (1968) reported reductions of over 50% in MetHb, when guinea pig blood was stored for a 24-h period in refrigerated conditions. The MetHb values reported here may not, therefore, represent the actual values at the time of sampling. However, no clinical signs of methemoglobinemia were observed during the experiment. Earlier reports also mentioned no clinical signs of methemoglobinemia when sheep were fed high doses of nitrate in their diets (Carver and Pfander, 1974; Alaboudi and Jones, 1985).

Feed Intake and Body Weight Gain

Feed intake of lambs in the adaptation period was not different among treatments. However, the small sample size of animals used in this study does not allow firm conclusions to be drawn concerning effects on feed intake. Bruning-Fann and Kaneene (1993) reported negative effects on feed intake in sheep when dietary nitrate exceeded 3% of DM. This reduction in feed intake may be related to a nitrite-induced depression of forage cell wall digestion as demonstrated in vitro by Marais et al. (1988). To avoid a possible reduction in DMI, sheep were fed restrictedly when in the respiration chambers.

Nitrate and Sulfate as Hydrogen Sinks

The inhibition of methane production by nitrate is most likely attributable to the energetically more favorable use of hydrogen in the reduction of nitrate to ammonia. This implies that 4 moles of hydrogen are redirected towards nitrate reduction, thereby theoretically lowering methane production by 1 mole for each mole of nitrate reduced, which is equivalent to a reduction of methane emission by 25.8 g for each 100 g of nitrate fed.

Sheep given nitrate in their diet consumed, on average, 25.2 g of nitrate/d during methane measurements, which would theoretically lower methane production by 6.5 g/d. The actual decrease in methane production for the nitrate treatment was 5.8 g/d. Thus, the decrease in methane production was 89% of the quantity that could be explained by stoichiometry, indicating that most of the nitrate fed was reduced in the rumen. The nitrate source used in this study was highly soluble and available in the rumen. Lewis (1951) found that approximately 8% of a nitrate load suddenly introduced directly into the rumen was recovered in urine. Irreversible loss of nitrate in urine may be responsible for the lower than predicted methane reduction in this study.

The addition of sulfate to the diet led to a reduction of 16% in daily methane production. Sulfate reduction to hydrogen sulfide also consumes 8 electrons and thus offers the same potential per mole to reduce methane emissions as nitrate. From a thermodynamic perspective, sulfate reduction is more favorable than methanogenesis (Ungerfeld and Kohn, 2006). Stoichiometrically, the full reduction of 100 g sulfate to hydrogen sulfide would reduce methane production by 16.7 g. In our study, sheep on the sulfate treatment consumed on average 25.8 g sulfate/d, which would correspond to a methane reduction of 4.3 g/d if it was all converted to H_2S. The actual observed decrease in methane was 2.9 g/day, which is 67% of the stoichiometrical potential.

The competition for hydrogen between sulfate reduction and methanogenesis has been studied in anaerobic digesters. Isa et al. (1986) concluded from their experiment that the extent of decrease in methane production from sulfate addition is dependent on both the sulfate concentration in the medium and the residence time within the digester. At low sulfate concentrations (0.5 g of sulfate/L) relatively more electrons were directed toward sulfate reduction compared with a high sulfate concentration (5.0 g of sulfate/L). Increasing the residence time from 0.5 to 10 d in the digester also markedly increased the flow of electrons from methanogenesis toward sulfate reduction. Rumen liquid passage rate in sheep is quite high (7-8%/h; Lopez et al., 2003), which might offer an explanation for the lower than expected use of hydrogen for sulfate reduction.

Sheep on sulfate treatments were fed a considerable amount of S in the diet (8.5 g of S/kg of DM). This level was chosen for two reasons: 1) to be able to quantify the contribution of sulfate reduction to reduce methane emissions, and 2) in an attempt to prevent nitrite accumulation and, therefore, problems associated with methemoglobinemia. The sulfate inclusion rate was, however, well above the maximum recommendations as indicated by NRC (4 g of S/kg of DM; NRC, 2001). Feeding above this upper limit on more traditional diets increases the risk of polioencephalomalacia, caused by high levels of hydrogen sulfide in the rumen headspace and the subsequent inhalation of hydrogen sulfide (Gould, 1998). Results from this experiment do, however, show that sulfate is effective in decreasing methane production. No clinical signs of polioencephalomalacia were observed during the experiment.

Effects of Nitrate and Sulfate on Oxygen Consumption and Carbon Dioxide and Heat Production

Oxygen consumption, carbon dioxide production and the resulting heat production were all lower in sheep consuming nitrate. Sheep on all treatments consumed the same amount of feed and gross energy during the heat production measurements. Therefore, the reduced heat production of the nitrate fed sheep results from a reduced metabolizability of the ingested gross energy, from a reduced conversion of ME into heat, potentially resulting in an increase in energy retention, or from a combination of the two. Because ME intake was not measured in this study, the distinction cannot be made. However, arguments for both options can be made.

It has been documented that accumulation of nitrite in rumen fluid reduces cell wall fermentation in vitro (Marais et al., 1988) and would therefore potentially reduce energy digestibility. In addition, reduced cell wall fermentability may explain the lower level of methanogenesis observed in this experiment for the nitrate treatment. As discussed previously, occurrence of methemoglobinemia could not fully be excluded in this study, and this may provide an explanation for a reduced conversion of ME into heat.

As concluded by Takahashi et al. (1998) oxygen consumption decreases by 1% for each 10% increase in methemoglobin. This would imply that in our study, methemoglobin would have been 6% of Hb in the nitrate-fed sheep and this may have gone undetected due to the time elapsed between sampling and analysis. On the other hand, potential depression of rumen digestion was not quantified in the study of Takahashi et al. (1998), nor in a subsequent study by Sar et al. (2005), and the reduction in oxygen consumption observed in these studies may, in part, be caused by a reduction in ME intake.

Surprisingly, sulfate feeding increased heat production. The S content in the control and nitrate diets were designed to be 1.2 g of S/kg of DM, which is below recommendations (NRC, 2001). Moreover, most of the dietary S was included in the formaldehyde-treated soybean meal, which probably resulted in a low rumen availability of S. Addition of S sources to S-deficient diets stimulates the total number of bacteria and stimulats rumen fermentation and microbial protein synthesis (Hegarty et al., 1994). Therefore, rumen fermentation may have been stimulated by the additional S from sulfate in the diet, resulting in an increased ME intake and a concomitant increase in heat production.

Effects of Nitrate and Sulfate on Ruminal Fermentation and Microbial Populations

The thermodynamically favorable reduction of nitrate preferentially directs hydrogen away from methanogenesis, but could also draw hydrogen away from other processes such as propionogenesis. Farra and Satter (1971) observed a shift in the VFA profile from propionate to acetate when diets high in nitrate were fed to dairy cows. The butyrate concentration was also significantly reduced. The same phenomenon was observed by Allison and Reddy (1984) when sheep were fed nitrates. No differences in concentrations of these VFA were observed in the present study. A major difference between the current experiment and that of Farra and Satter (1971) is the time of rumen fluid sampling. Farra and Satter (1971), using fistulated cows, sampled 1 h after feeding, whereas our samples were obtained approximately 24 h after feeding. The methane-lowering effect was only apparent in the 12-h period after feeding (Figure 1) and the same may be true for any effects on VFA production, Because both are dependent on the competition for hydrogen in nitrate reduction. In sheep, a shift in VFA proportions from butyrate to acetate was reported when nitrate was included in the diet (Alaboudi and Jones, 1985). However, this shift was only observed up to 1.5 h after feeding. The addition of nitrate to the diets invoked a decrease in the proportion of BCVFA in this experiment. This may reflect a lower level of dietary proteolysis or reduced microbial lysis on this treatment. Rumen fluid of sheep with added sulfate in their diet contained a higher proportion of BCVFA, which may be related to the higher microbial activity on this treatment.

Dietary addition of nitrate decreased the number of methanogens in the rumen fluid of these sheep, which may be attributed to a lower electron pool available to the methanogens or to the toxicity of the intermediately formed nitrite during nitrate reduction (Allison et al., 1981). Methanogens are dependent on hydrogen availability and depletion of hydrogen through uptake by nitrate-reducing bacteria may explain the declining population density of methanogens. The lower number of methanogens observed on the nitrate treatment might be an alternative explanation for the reduced methane production observed for this treatment.

CONCLUSIONS

Adding salts of nitrate or sulfate to the diet of sheep reduced enteric methane production. Moreover, the effects of both products on methane production were additive. Provided that these substances can be fed in a safe way, they are powerful agents to reduce methane production by sheep.

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CHAPTER 5

Persistency of methane mitigation by dietary nitrate supplementation in dairy cows

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ABSTRACT

Feeding nitrate to dairy cows may lower ruminal methane production by competing for reducing equivalents with methanogenesis. Twenty lactating Holstein-Friesian dairy cows (33.2 ± 6.0 kg of milk/d; 104 ± 58 d in milk at the start of the experiment) were fed a total mixed ration (corn silage-based; forage to concentrate ratio 66:34), containing either a dietary urea or a dietary nitrate source [21 g of nitrate/kg of dry matter (DM)] during 4 successive 24-d periods, to assess the methane-mitigating potential of dietary nitrate and its persistency. The study was conducted as paired comparisons in a randomized design with repeated measurements. Cows were blocked by parity, lactation stage and milk production at the start of the experiment. A 4-wk adaptation period allowed the rumen microbes to adapt to dietary urea and nitrate. Diets were isoenergetic and isonitrogenous. Methane production, energy balance, and diet digestibility were measured in open-circuit indirect calorimetry chambers. Cows were limit-fed during measurements. Nitrate persistently decreased methane production by 16%, either expressed in grams per day, grams per kilogram of dry matter intake (DMI) or as percentage of gross energy intake, which was sustained for the full experimental period (mean 368 vs. 310 ± 12.5 g/d; 19.4 vs. 16.2 ± 0.47 g/kg of DMI; 5.9 vs.4.9 ± 0.15 % of gross energy intake for urea vs. nitrate, respectively). This decrease was smaller than the stoichiometrical methane mitigation potential of nitrate (full potential = 28% methane reduction). The decreased energy loss from methane resulted in an improved conversion of dietary energy intake into metabolizable energy (57.3 vs. 58.6 ± 0.70%, urea vs. nitrate, respectively). Despite this, milk energy output or energy retention was not affected by dietary nitrate. Nitrate did not affect milk yield or apparent digestibility of crude fat, neutral detergent fiber and starch. Milk protein content (3.21 vs. 3.05 ± 0.058%, urea vs. nitrate respectively), but not protein yield, was lower for dietary nitrate. Hydrogen production between morning and afternoon milking was measured during the last experimental period. Cows fed nitrate emitted more hydrogen. Cows fed nitrate displayed higher blood methemoglobin levels (0.5 vs. 4.0 ± 1.07% of hemoglobin, urea vs. nitrate respectively) and lower hemoglobin levels (7.1 vs. 6.3 ± 0.11 mmol/L, urea vs. nitrate respectively). Dietary nitrate persistently decreased methane production from lactating dairy cows fed restricted amounts of feed, but the reduction in energy losses did not improve milk production or energy balance.

INTRODUCTION

The production of ruminant meat and milk is associated with a relatively high production of greenhouse gases (GHG) compared with other food commodities (Williams et al., 2008). In a recent publication, the contribution of the dairy sector to the global production of greenhouse gases was estimated to be 2.7% (FAO, 2010). This high level of GHG production is mainly related to the anaerobic fermentation of fiber-rich feedstuffs in the gastrointestinal tract of ruminants. Methane production from enteric fermentation accounts for 52% of the carbon footprint of milk, expressed in CO_2-equivalents, at the farm gate (FAO, 2010). A decrease in the amount of enteric methane could substantially decrease the amount of GHG associated with milk production. Although many dietary strategies have been proposed to reduce methane production from ruminants (Martin et al., 2010), few have shown a persistent decrease of methane production in vivo. Persistency of the methane-decreasing effect is an absolute requirement for any dietary strategy to be successful in abating GHG emissions from ruminants.

Most methane production during rumen fermentation is the result of the reduction of CO_2 with H_2 by methanogenic Archaea residing in the rumen. This process enables the removal of excess H_2 from the rumen and allows NADH to be reconstituted to NAD^+, a process essential to the continuation of anaerobic rumen fermentation and microbial growth (Wolin, 1975).

Anaerobic nitrate reduction is energetically more favorable than CO_2 reduction, and the presence of nitrate in the rumen redirects H_2 from methanogenesis to nitrate reduction, thereby decreasing methane production (Allison and Reddy, 1984). However, the sudden introduction of nitrate into ruminant diets may lead to the occurrence of methemoglobinemia, a condition caused by the oxidation of the ferric iron in hemoglobin, rendering the molecule incapable of oxygen transport. The oxidation of hemoglobin is caused by the presence of nitrite, an intermediate in nitrate reduction, in blood. Gradual introduction of nitrate into the diet can allow the rumen microbes to adapt and increase their capability to reduce nitrite (Alaboudi and Jones, 1985). Van Zijderveld et al. (2010), using a 4-wk adaptation period to dietary nitrate, demonstrated decreased methane emissions (-32%) in sheep fed nitrate, while methemoglobinemia was not observed. Similarly Nolan et al. (2010) used an 18-d adaptation period and observed 23% decrease in methane emissions in sheep fed nitrate, again without the occurrence of methemoglobinemia. The effect of feeding nitrate to dairy cows on enteric methane production has not yet been investigated. In addition, it is unknown if the methane-depressing effect of nitrate persists over time.

This experiment was designed to investigate to what extent dietary nitrate can decrease enteric methane production in lactating dairy cows and if this would affect animal productivity, feed digestibility, or energy balance. After a 4-wk adaptation period, methane production was measured in 4 consecutive periods with 24-d intervals to investigate if the methane decrease caused by dietary nitrate was persistent.

MATERIALS AND METHODS

Experimental Design

The Animal Care and Use Committee of Wageningen University (Wageningen, the Netherlands) approved the experimental protocol. The experiment was designed as a completely randomized block with repeated measurements. Twenty Holstein-Friesian dairy cows were blocked according to parity, lactation stage, and milk production at the start of the experiment and subsequently within a block randomly allocated to 1 of 2 diets. One diet (hereafter, the nitrate diet) contained a nitrate source (Calcinit; Yara, Oslo, Norway) which was replaced by urea, on an isonitrogenous basis, in the other diet (the urea diet).

Animals and Housing

The experiment was conducted at the experimental dairy farm of Wageningen University and Research (Wageningen, The Netherlands). The experimental group included 20 lactating Holstein-Friesian dairy cows (initial milk production 33.2 ± 6.0 kg; 104 ± 58.0 DIM). During a 4-wk adaptation period, cows were housed in a freestall dairy barn in 2 separate treatment groups. The adaptation period served to introduce the experimental concentrates gradually into the diet.

After adaptation to the diet, cows were subjected to a 17-d experimental period. During this period, cows were housed in tie-stalls for 12 d and subsequently moved to respiration chambers for a period of 5 d. In the tie-stalls, animals were accustomed to be restricted in movement, and individual feed intake was measured. In the respiration chambers, gaseous exchange, individual feed intake, and feed digestibility were determined. The 17-d experimental period was replicated 4 times for each pair of animals with 24-d intervals between measurements to evaluate persistency of effects. During the 7 d cows were not in the tie-stalls, they were housed in a freestall dairy barn, but were maintained in their treatment groups, and fed their respective treatment diets. In this publication, d 1 refers to the first day cows were housed in the tie-stalls. Only 2 respiration chambers were available for implementation of this experiment, which limited the number of animals that could be measured at the same time to 4 (2 on each treatment; 2 cows per chamber) and the groups of cows had to be allocated to the chambers in a staggered manner. Because of this allocation, the first group of cows completed the experiment after 89 d on the full nitrate diet, whereas the last group of cows had been on the full nitrate diet for 107 d.

Diets and Feeding

Both diets consisted of 53% corn silage, 9% dried alfalfa, 4% barley straw and 34% concentrates on a DM basis. The diets were offered as TMR and were prepared on a daily basis using a mixer wagon (Verti-mix 500, Strautmann, Bad Laer, Germany). The concentrates for the urea and nitrate diets were balanced for N and Ca by the isonitrogenous exchange of urea for nitrate and the addition of limestone to the urea diet concentrate, respectively. Before the start of the adaptation period, the concentrate portion of the diet consisted of adaptation concentrates (*Table 5.1*). At the start of the adaptation period, 25% of the adaptation concentrates were replaced by 1 of the 2 experimental concentrates (urea or nitrate) for each treatment.

Table 5.1

Ingredient composition (% of DM) of concentrates containing either urea or nitrate as NPN source

	Adaptation	Urea	Nitrate
Formaldehyde-treated soybean meal	-	31.8	31.8
Soybean meal	51.3	0.0	0.0
Wheat	6.3	19.0	19.0
Corn	12.8	12.7	12.7
Dried beet pulp	20.1	13.3	13.3
Fractionated palm oil	3.7	7.3	7.3
Trace mineral and vitamin premix	2.2	2.2	2.2
Monocalciumphosphate	1.5	2.0	2.0
$MgSO_4$	-	1.5	1.5
NaCl	0.7	1.5	1.5
Urea	1.5	3.5	-
Wood cellulose	-	0.3	-
$CaCO_3$	-	5.1	-
Nitrate source[1]	-	-	8.8

[1] $5Ca(NO_3)_2 \cdot NH_4NO_3 \cdot 10H_2O$; 75% NO_3 in DM

This ration was fed for 1 wk, after which the proportion of experimental concentrates was increased to 50% of the total amount of concentrates. Dietary proportions of experimental concentrates were increased by 25% in each additional week of the adaptation period, until the concentrate proportion of the diets consisted fully of experimental concentrates after 3 wk. A fourth week was added to the adaptation period when the full amount of experimental concentrates was fed. The 4-wk period served to allow adaptation of the rumen microflora and to allow sufficient time for increased activity of bacterial nitrate and nitrite reductases (Alaboudi and Jones, 1985).

Cows were group-fed during the adaptation period. Cows were fed once daily during the period in the freestall barn (0900 h) and twice daily (0630 h and 1630 h; equal portions) during each 17-day experimental period. Water was freely available during the entire experiment.

In the tie-stalls and respiration chambers cows were individually fed. Orts were collected daily. Cows were fed ad libitum for the first 8 d of the experimental period and subsequently restricted in feed intake for the remainder of the 17-d experimental period. Within a block, feed intake was restricted to 95% of the ad libitum intake of the animal consuming the lowest amount of feed during d 5 to 8. This approach was chosen to ensure similar feed intake between treatments, thus avoiding confounding effects of DMI on methane production.

Sampling and Chemical Analysis

Representative samples of TMR (±500 g) were collected at each preparation of fresh TMR and stored frozen (-20 °C) pending analysis. Samples were thawed, pooled per

period and treatment, subsampled (±500 g), freeze-dried and ground to pass a 1-mm screen before analysis. Individual roughage and concentrate samples were taken on d 10 of each period and stored frozen. After the experiment, corn silage, straw, and alfalfa samples were analyzed by near infrared spectroscopy (Blgg, Oosterbeek, the Netherlands). Samples of dried alfalfa and straw were pooled by roughage type over the entire experiment and subsampled before analysis (1 sample for each roughage type); corn silage samples were analyzed individually per measuring period. The total production of feces and urine was collected after completion of each measurement period, mixed thoroughly, and subsampled for analysis. Dry matter, CP, crude fat, sugar, starch and NDF content of TMR, concentrate and manure samples were determined according to the methods described in detail in Abrahamse et al. (2008). Gross energy (GE) content was determined using bomb calorimetry (IKA-C700, Janke & Kunkel, Heitersheim, Germany). Nitrate was determined as described previously by van Zijderveld et al. (2010).

Gaseous Exchange and Diet Digestibility

The respiration indirect calorimetric chambers used in this study have been described in detail by Verstegen et al. (1987). Measurements of gaseous exchange and diet digestibility were performed as described previously by van Zijderveld et al (2011). Heat production was calculated according to the methods of Brouwer (1965). Cows were housed in pairs on the same treatment in the respiration chambers.

Determination of Hydrogen Production

Hydrogen production was determined in period 4 of this experiment between 0630 h and 1530 h. Air samples were taken manually from the in- and outgoing air from the respiration chambers by means of a syringe (2-3 samples/h). The syringe was injected into a gas chromatograph (Quintron Breathtracker DP, Quintron Instrument Company, Milwaukee, WI) within approximately 10 min after the sample was taken. Hydrogen production was subsequently calculated by multiplication of the H_2 concentrations by the measured air flow rate through the chambers.

Milk Yield and Composition

Cows were milked twice daily during the entire experiment (0630 and 1630 h). Milk yield was determined daily. In the respiration chamber, milk was sampled during each milking and stored in a refrigerator at 4°C in tubes containing sodium azide. These samples were analyzed for fat, CP, lactose, urea and SCC as described previously by van Zijderveld et al. (2011) at the end of each experimental period. Average milk component concentrations during the measurement period were calculated from the weighted average of all samples taken. At each milking, additional samples (3 g/kg of milk) were taken for GE determination.

Blood sampling

In the respiration chambers, blood was sampled on the third day of each measurement period. Blood was sampled from the tail vein at 3 h post feeding in heparinized collection tubes (Becton Dickinson, Breda, The Netherlands). Blood was analyzed for

hemoglobin (Hb) and methemoglobin (MetHb) contents within 1 h after collection with a hemoximeter (OSM 3, Radiometer, Copenhagen, Denmark).

Statistical Analyses

Data collected during the measurement period only were used for statistical analyses. Data on gaseous emissions from the last 72 h of each measurement period were averaged per period before analysis. Data measured on individual animals (milk production, milk composition, blood parameters and DMI) were subjected to repeated-measures ANOVA including block and treatment as fixed factors. Data collected during the 4 experimental periods were treated as repeated measures per animal or pair of animals. Data collected on pairs of cows (gaseous exchange and diet digestibility parameters) were subjected to repeated-measures ANOVA with treatment as fixed factor. Significance of effects was declared at $P < 0.05$. The statistical program Genstat (11th ed., Lawes Agricultural Trust, Rothamsted, UK) was used to analyze the data.

RESULTS

Diet Composition

Chemical composition of dietary concentrates was established as formulated (*Table 5.2*); complete diets were iso-energetic (on a GE basis) and isonitrogenous.

Table 5.2

Analyzed chemical composition of TMR ingredients and complete TMR containing either a urea or nitrate source

	TMR ingredient					TMR	TMR
	Corn silage	Alfalfa	Barley straw	Concentrates		Urea	Nitrate
				Urea	Nitrate		
Inclusion (% of DM)	53	9	4	34	34		
Gross energy (MJ/kg of DM)	NA[1]	NA	NA	NA	NA	18.7	18.7
DM (g/kg)	365	881	894	899	884	520	523
Crude ash (g/kg DM)	42	67	67	133	134	77	76
CP (g/kg DM)	74	114	28	318	317	156	156
Crude fat (g/kg DM)	32	NA	NA	96	92	44	43
NDF (g/kg DM)	402	531	783	112	116	329	325
Starch (g/kg DM)	327	NA	NA	259	259	234	236
Nitrate (g/kg DM)	0	0	0	0	63	0	21

[1] NA = not analyzed

Animal Performance and Methane Production

Methane production was reduced by 16% on the nitrate diet (*Table 5.3*) when expressed on a daily basis, as grams per kilogram of DMI or as percentage of gross energy intake (GEI). When expressed per kilogram of milk, methane was decreased by 14%. Daily methane production increased with time during the experiment for both treatments. No significant interaction was observed between treatment and time.

Dry matter intake was not affected by treatment, but increased with time for both treatments. Milk yield was unaffected by treatment, but decreased in time as cows advanced in lactation. Protein content of milk was lower for the nitrate diet (3.21 vs. 3.05 ± 0.058%, urea vs. nitrate respectively), but milk protein yield was unaffected by treatment. Other milk constituents were unaffected by treatment or time.

Energy Balance, Nitrogen Balance, and Digestibility

Gross energy intake was not affected by treatment, but increased as the experiment progressed (*Table 5.4*). Energy lost in methane production was lower for the nitrate diet and increased during the experiment for both treatments. The energy lost in urine and feces and ME intake (MEI) were unaffected by treatment. Although MEI was not affected by treatment, the MEI:GEI ratio was elevated as a consequence of nitrate feeding (57.3 vs. 58.6 ± 0.70%, urea vs. nitrate, respectively). Calculated heat production was unaffected by treatment, but increased over time. Milk energy yield was unaffected by treatment or time. Despite the higher MEI:GEI ratio, energy retention (ER) was not increased for the nitrate treatment. Apparent total-tract digestibility of NDF, starch and crude fat were unaffected by treatment.

Nitrogen balance (*Table 5.5*) was mostly positive for both treatments and no differences between treatments were observed. Nitrogen intake increased as the experiment progressed. The conversion efficiency of feed N into milk N was not affected by treatment but decreased with time for both treatments.

Table 5.3

Dry matter intake, milk production, milk composition, and methane production of limit-fed dairy cows fed either a urea source or a nitrate source

	d 13 – d 17		d 37 – d 41		d 61 – d 65		d 85 – d 89		Pooled	P-value[1]		
	Urea	Nitrate	Urea	Nitrate	Urea	Nitrate	Urea	Nitrate	SEM	Trt	Time	Trt x time
DMI (kg/d)	17.9	18.0	19.1	19.7	19.4	19.2	19.7	19.7	0.76	0.877	< 0.001	0.402
Milk production (kg/d)	27.9	28.3	27.4	28.3	26.2	26.6	25.5	26.6	0.87	0.452	0.009	0.877
FPCM[2] (kg/d)	28.1	27.7	27.9	27.9	26.6	26.4	25.7	26.4	0.91	0.990	0.020	0.814
Fat (%)	4.27	4.02	4.28	4.02	4.24	4.07	4.19	4.04	0.172	0.353	0.947	0.900
Protein (%)	3.09	2.95	3.24	3.05	3.25	3.09	3.24	3.09	0.058	0.041	0.001	0.902
Fat (g/d)	1165	1133	1152	1132	1097	1079	1052	1077	71.6	0.852	0.050	0.782
Protein (g/d)	860	834	884	864	848	821	821	823	27.4	0.547	0.138	0.861
Lactose (%)	4.47	4.48	4.47	4.53	4.46	4.53	4.46	4.49	0.058	0.623	0.285	0.311
SCC (x 1000 cells/mL)	82	120	231	108	94	108	75	111	40.7	0.799	0.202	0.172
MUN (mg/dL)	12.1	12.4	12.0	11.9	13.2	12.3	12.2	12.3	0.49	0.735	0.176	0.334
CH_4 (g/cow per day)	341	283	371	313	378	318	383	326	12.5	0.009	< 0.001	0.961
CH_4 (g/kg of DMI)	19.1	15.8	19.6	15.9	19.5	16.6	19.5	16.5	0.47	< 0.001	0.041	0.313
CH_4 (g/kg of milk)	11.8	10.4	13.3	11.5	13.9	11.9	15.0	12.4	0.52	0.010	< 0.001	0.449
CH_4 (% of GEI[3])	5.7	4.7	5.7	4.7	6.1	5.1	6.1	5.1	0.15	< 0.001	< 0.001	0.817

[1] Trt = treatment.
[2] Fat- and protein-corrected milk.
[3] Gross energy intake.

Table 5.4

Energy balance and apparent total-tract diet digestibility of limit-fed dairy cows fed either a urea source or a nitrate source

	d 13 – d 17		d 37 – d 41		d 61 – d 65		d 85 – d 89		Pooled	P-value[1]		
	Urea	Nitrate	Urea	Nitrate	Urea	Nitrate	Urea	Nitrate	SEM	Trt	Time	Trt x time
Metabolic BW (kg/cow)	117	120	118	121	122	118	121	122	3.2	0.912	0.273	0.132
GEI[2] (kJ/kg of $BW^{0.75}$ per day)	2831	2789	3033	3060	2834	2930	2904	2887	75.5	0.863	0.002	0.433
Energy feces + urine (kJ/kg of $BW^{0.75}$ per day)	1023	1012	1108	1112	1086	1077	1058	1053	35.2	0.904	0.003	0.983
Methane production (kJ/kg of $BW^{0.75}$ per day)	161	131	174	143	172	149	176	148	5.0	0.002	< 0.001	0.425
MEI[3] (kJ/kg of $BW^{0.75}$ per day)	1647	1645	1750	1805	1577	1704	1670	1686	49.0	0.375	0.015	0.332
MEI:GEI ratio (%)	58.2	59.0	57.7	59.0	55.6	58.2	57.5	58.3	0.70	0.036	0.118	0.498
Heat production (kJ/kg of $BW^{0.75}$ per day)	951	962	989	998	975	987	987	991	17.9	0.713	< 0.001	0.929
Energy in milk (kJ/kg of $BW^{0.75}$ per day)	704	710	702	700	715	691	656	697	18.1	0.699	0.269	0.290
ER[4] total (kJ/kg of $BW^{0.75}$ per day)	-9	-27	60	107	-114	26	29	-3	49.9	0.433	0.105	0.275
ER protein[4] (kJ/kg of $BW^{0.75}$ per day)	0	22	8	17	11	22	28	33	11.6	0.247	0.287	0.799
ER fat[4] (kJ/kg of $BW^{0.75}$ per day)	-9	-48	52	90	-124	4	1	-36	43.1	0.558	0.051	0.180
NDF digestibility (%)	52.1	52.7	52.6	53.9	52.1	53.9	53.3	52.7	1.09	0.364	0.846	0.672
Starch digestibility (%)	97.3	96.6	98.0	98.5	97.5	98.7	98.4	98.5	0.28	0.217	0.002	0.053
Fat digestibility (%)	70.2	71.8	60.4	66.3	63.1	57.9	64.8	59.6	3.19	0.791	0.027	0.217

[1] Trt = treatment.
[2] GEI = gross energy intake.
[3] MEI = ME intake; MEI = GEI – energy feces + urine – methane production.
[4] ER = energy retention. ER total = MEI – heat production – energy in milk; ER protein = protein gain × 23.6 kJ/g of protein; ER fat = ER total – ER protein.

Table 5.5

Nitrogen balance (mg/kg $BW^{0.75}$ per day) and N efficiency of limit-fed dairy cows fed either a urea source or a nitrate source

	d 13 – d 17 §		d 37 – d 41		d 61 – d 65		d 85 – d 89		Pooled	P-value[1]		
	Urea	Nitrate	Urea	Nitrate	Urea	Nitrate	Urea	Nitrate	SEM	Trt	Time	Trt x time
N intake	3814	3829	3974	4037	4032	3981	4050	4064	103.2	0.935	0.024	0.819
N urine + feces	2573	2509	2707	2734	2746	2680	2749	2680	80.2	0.660	0.009	0.729
N milk	1148	1080	1154	1122	1145	1078	1052	1091	28	0.107	0.189	0.242
N condens[2]	92	95	60	66	70	74	61	65	21.6	0.794	0.360	0.978
N balance	1	146	53	114	72	149	189	226	78.7	0.247	0.287	0.799
N efficiency (N milk/N feed)	30.8	28.9	29.6	28.5	29.2	27.7	26.6	27.4	0.98	0.267	0.073	0.450

[1] Trt = treatment.
[2] N in condensate that was collected from the heat exchanger in the respiration chamber

Table 5.6

Hemoglobin (Hb) and methemoglobin (MetHb) levels of limit-fed dairy cows fed either a urea source or a nitrate source

	d 13 – d 17		d 37 – d 41		d 61 – d 65		d 85 – d 89		Pooled	P-value[1]		
	Urea	Nitrate	Urea	Nitrate	Urea	Nitrate	Urea	Nitrate	SEM	Trt	Time	Trt x time
Hb (mmol/L)	6.9	6.1	7.0	6.3	7.4	6.3	7.2	6.5	0.11	< 0.001	0.003	0.147
MetHb (% of Hb)	0.4	4.2	0.4	3.6	0.5	3.5	0.6	4.7	1.07	0.008	0.814	0.869
MetHb minimum (% of Hb)	0.1	0.6	0.2	0.6	0.2	0.7	0.5	0.7	-	-	-	-
MetHb maximum (% of Hb)	0.8	14.5	0.6	19.0	0.7	7.9	0.8	15.3	-	-	-	-

[1] Trt = treatment.

Hydrogen Production

Cumulative hydrogen production was higher for the nitrate diet during the 9-h measurement period in experimental period 4 (1.9 L/cow per 9 h for urea vs. 5.8 L/cow per 9 h for nitrate; P = 0.003). Hydrogen production was higher up to 7 h after feeding for the nitrate diet (Figure 5.1). The variability in hydrogen production was much larger for pairs of animals on the nitrate treatment than those on the urea treatment. Methane production was suppressed for a period of 5 h after feeding with nitrate, and this depression in methane production largely coincided with the increase in hydrogen production for this treatment.

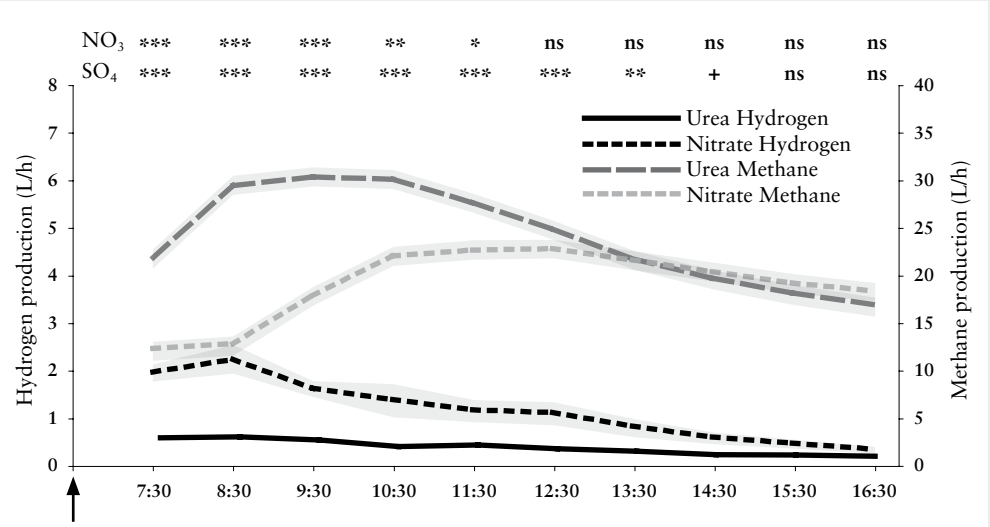

Figure 5.1

Ruminal hydrogen and methane production over a 9-h period from lactating dairy cows fed either a urea or nitrate source.

Data represent mean values obtained during experimental period 4 of the main experiment. Arrow indicates time of feeding. Superscripts indicate whether the treatment effect was significant for the respective time point (*** = P< 0.001; ** = P< 0.01; * = P < 0.05' + = P < 0.10; ns = not significant)

Blood Parameters

Methemoglobin content of blood was consistently higher for cows on the nitrate diet (*Table 5.6*) and was not affected by time on treatment. Hemoglobin content was lower for the nitrate-fed animals and increased with time for both treatments.

DISCUSSION

Methane Abatement by Dietary Nitrate

In this research, we demonstrated for the first time that nitrate decreases methane production persistently in dairy cows. The methane decrease confirms earlier observations in nitrate-fed sheep (Nolan et al., 2010; van Zijderveld et al., 2010) or sheep ruminally infused with nitrate solutions (Takahashi and Young, 1991; Sar et al., 2004). We hypothesized that reduction of nitrate to nitrite and then ammonia would provide a sink for metabolic hydrogen, thus decreasing its availability for methanogenesis. Nitrate reduction is energetically more favorable than methanogenesis (Ungerfeld and

Kohn, 2006). Cows in this experiment consumed, on average, 21 g of nitrate/kg of DM. Complete reduction of this nitrate to ammonia would consume sufficient hydrogen to decrease methane emissions by 5.4 g methane/kg of DM (25.8 g of CH_4 decrease/100 g of nitrate fed; van Zijderveld et al, 2010). The decrease in methane production was only 3.2 g of methane/kg of DM or 59% of this theoretical potential. This could indicate that nitrate was not fully reduced to ammonia, using only part of the theoretical amount of hydrogen required compared with a situation where full reduction would have occurred. Considering that hydrogen losses also increased with the nitrate diet, the actual efficiency of nitrate reduction in abating methanogenesis would be even lower.

Cows in both treatment groups were fed a diet containing 156 g of CP/kg of DM. Twenty-two percent of this CP consisted of NPN, originating from either urea or nitrate. Rumen microbes require a minimum level of rumen ammonia to maintain optimal growth conditions (Dijkstra et al., 1998). If 44% of the nitrate would not have been reduced to ammonia, this would theoretically have lowered the available CP content of the nitrate diet by 15 g/kg of DM, mainly limiting availability of rumen-available N. When calculated according to the Dutch protein evaluation (DVE) system, the decreased availability of N from nitrate would have resulted in a negative rumen available N balance, which could be expected to have a negative impact on microbial protein synthesis and dairy cow productivity. Milk CP concentration was lower for the nitrate diet; however, daily milk protein production was unaffected by treatment and the lower protein concentration may have been a consequence of dilution. In addition, the efficiency with which N was used for milk protein synthesis was unaffected by treatment. Moreover, MUN levels did not differ between treatments; if ammonia levels in the rumen were lower because of incomplete reduction of nitrate, this probably would have been reflected in decreased MUN levels (Gustafsson and Palmquist, 1993). It therefore seems unlikely that incomplete nitrate reduction was responsible for the lower relative effectiveness of nitrate in dairy cows.

Methanogenesis is not the only sink for hydrogen in the rumen. Hydrogen is also used for the production of the more reduced acids propionate and valerate (Czerkawski, 1972), and increases in molar proportions of propionate in rumen fluid are negatively associated with methanogenesis (Ellis et al., 2008; Janssen, 2010). Nitrate reduction is thermodynamically more favorable than propionogenesis (Ungerfeld and Kohn, 2006) and may successfully compete for hydrogen with propionogenesis. This would mean that nitrate reduction draws electrons from both methanogenesis and propionogenesis. Indeed, it has been demonstrated that feeding nitrate at 2% of DM to dairy cows increases the molar proportion of acetate, at the expense of propionate and butyrate (Farra and Satter, 1971), and such a shift will decrease the amount of hydrogen consumed in propionogenesis (Ellis et al., 2008). The efficacy of nitrate as a methane inhibitor may therefore depend on the relative importance of propionogenesis in the host animal.

In sheep, the apparent efficiency of nitrate use in methane mitigation was much higher (78%, Nolan et al, 2010; 89%, Van Zijderveld et al, 2010) than in cattle in the present experiment (59%). Sheep have a much lower feed intake relative to their body weight and a higher rumen pH; in that situation, relatively less propionic acid and more acetic acid and butyric acid are produced (Bannink et al., 2008). Therefore, propionogenesis might play a smaller role in sheep compared with lactating cows and nitrate reduction

might obtain more of its reducing equivalents in competition with propionogenesis in dairy cows and more in competition with methanogenesis in sheep. This would imply that nitrate is more effective in decreasing methane emissions in animals fed near maintenance, with a relatively minor role for propionogenesis.

It is likely that part of the ingested nitrate, and part of the nitrite formed in the rumen, were absorbed through the rumen wall into the blood and subsequently lost in urine (Takahashi et al., 1998) and thus unavailable for reduction to ammonia in the rumen. Clearance of nitrate and nitrite to blood might therefore be an additional explanation for the lower than expected inhibition of methanogenesis. In previous studies with sheep (Nolan et al., 2010; Van Zijderveld et al., 2010) the dietary inclusion of nitrate was higher than in the current study (24 and 26 g of nitrate/kg of DM in the respective sheep studies compared with 21 g of nitrate/kg of DM in the current study). However, when expressed relative to metabolic BW, the nitrate dose in the current experiment was considerably higher than in our previous study with sheep (3.4 g of nitrate/kg of $BW^{0.75}$ per day vs. 1.6 g of nitrate/kg of $BW^{0.75}$ per day for dairy cows and sheep, respectively). This higher dose may have led to a higher proportion of the dietary nitrate entering the blood through the rumen wall and being lost in urine. This could be another explanation for the relatively higher efficiency of methane abatement with dietary nitrate in sheep.

Cows on the nitrate diet had elevated blood MetHb levels. This indicates that a pool of nitrite, absorbed from the rumen, was present in the blood. In sheep studies (Nolan et al., 2010; van Zijderveld et al., 2010), no significant elevations of MetHb were observed when similar levels of dietary nitrate on diet DM basis were fed. This supports the above-mentioned hypothesis that larger amounts of nitrate and nitrite are transferred from the rumen to the blood when higher doses, expressed per kilogram of metabolic weight, are fed.

Methane is a loss of dietary energy for the dairy cow (Johnson and Johnson, 1995), and the capture of lost hydrogen may be a way to enhance the energetic efficiency of the cow. In our experiment, the reducing equivalents that would normally have been lost in methane production are assumed to have been taken up during nitrate reduction to ammonia. This might have benefited animal productivity if ruminal ammonia concentrations had been limiting animal production. This is unlikely, however; dietary CP levels were adequate on both treatments and MUN levels suggest that dietary N was not limiting productivity. Moreover, nitrate reduction may have even drawn reducing equivalents away from propionogenesis in our experiment. Propionate may be a nutrient limiting animal productivity (Huhtanen et al., 1998) and the redirection of reducing equivalents into ammonia rather than propionate may even be more limiting to animal productivity. In the present experiment, although methane production was reduced and the ratio of MEI to GEI increased, energy in milk or energy retention was not affected by nitrate supplementation.

Occurrence of Methemoglobinemia

Methemoglobinemia occurs when nitrite is absorbed from the rumen into the blood of the animal. Ferrous iron (Fe^{2+}) in hemoglobin is transformed into ferric iron (Fe^{3+}), rendering

the hemoglobin molecule (now called methemoglobin) incapable of transporting oxygen to the tissues (Ozmen et al., 2005). Nitrite is formed in the rumen as an intermediate in the reduction of nitrate to ammonia. In animals not previously adapted to nitrate in their diet, the reduction nitrate to nitrite occurs at a higher rate than the reduction of nitrite to ammonia, resulting in an accumulation of nitrite in the rumen and subsequent absorption (Bruning-Fann and Kaneene, 1993). Adapting animals slowly to nitrate in their diet enables the population of nitrite-reducing bacteria to increase in size, increasing the capacity to reduce nitrite (Allison and Reddy, 1984). An adaptation period has been demonstrated to enable ruminants to cope with higher levels of nitrate in their diets (Alaboudi and Jones, 1985; van Zijderveld et al., 2010). In our study an adaptation period to dietary nitrate was included, but MetHb levels were still higher (P = 0.008; *Table 5.6*) for the nitrate-fed cows. It has been proposed that animals respond to prolonged elevated MetHb levels in their blood by producing more Hb, thereby compensating for the decreased oxygen-carrying capacity of the blood (Winter and Hokanson, 1964). No evidence of such a mechanism was observed in this study. Although average MetHb levels were no reason for concern on the nitrate diet, peak levels for individual animals were 4- to 5-fold the average level in the cows on the urea diet, but still below the level considered to cause subclinical methemoglobinemia (30-40%; Bruning-Fann and Kaneene, 1993)

Yield of Usable Metabolizable Energy as a Consequence of Methane Reduction

If GEI and digestibility are not altered, a reduction in methane production will increase the amount of ME available to the animal, which could increase milk production or body tissue gain (Blaxter and Czerkawski, 1966). In this experiment, methane production was lowered (-28 kJ/kg of $BW^{0.75}$ per day, or 3.4 MJ/d) and apparent NDF, crude fat and starch digestibility were unaffected by treatment. The conversion of GE into ME was also improved for the nitrate diet. If the energy spared from methanogenesis had been fully converted into milk with an efficiency of 0.64 (NRC, 2001), the increase in milk yield would have been 0.7 kg (milk energy was 3.1 MJ/kg for this experiment). However, no significant increase in milk energy output was observed in this experiment, although milk energy output was numerically higher for the nitrate diet (+6 kJ/kg of $BW^{0.75}$ per day, or 0.7 MJ/d). The majority of the energy saved from a lower methane production appeared to accumulate in the calculated energy retention which increased nonsignificantly for the nitrate diet (+34 kJ/kg of $BW^{0.75}$ per day, or 4.1 MJ/d). Despite the significant reduction in methane production, no positive responses in animal performance parameters were observed.

During experimental period 4, hydrogen production was measured and was found to be higher for the nitrate diet. Our hypothesis was that nitrate would act as a hydrogen sink and the reason for the elevated hydrogen emissions for the nitrate diet is unknown. Hydrogen is an energy-dense gas and its emission by the animal could offset the energy benefit gained by the decrease in methane production. In experimental period 4, hydrogen production was measured from 0630 h to 1530 h. The average hydrogen production over this 9-h period was 1.9 L/cow for the urea diet and 5.8 L/cow for the nitrate diet. If results from the 9-h period are extrapolated to a 24-h period, hydrogen production is estimated to amount to 5 L/cow per day for the urea diet and 15 L/cow per day for the nitrate diet. The additional 10 L/d of hydrogen produced for the nitrate diet translate into 0.45 mol

of hydrogen/d (22.4 L/mole) or 0.9 g of hydrogen/d. Hydrogen is energy dense (142 kJ/g of H_2; Afeefy et al., 2011), but the additional energy lost in hydrogen production for the nitrate diet was calculated to be relatively minor (approximately 1.0 kJ/kg $BW^{0.75}$ per day or 3.6% of the observed methane decrease).

The simple statement that reducing enteric methane emissions without affecting feed digestibility will increase the animals' productivity or energy retention therefore appears to be inadequate. A further requirement would be that the reducing equivalents spared from methane production are incorporated in molecules that are limiting either bacterial or animal metabolic processes. The direct environmental impact of methane production from the dairy cows in this experiment was decreased; daily methane production was lowered by 58 g/cow per day, or 1.5 kg of CO_2-equivalents/cow per day.

Conclusions

Nitrate addition to corn silage-based dairy cow diets for 89 d persistently decreased enteric methane emissions by 16% without negatively affecting diet digestibility and milk production. The energetic benefit from the decreased methane production did not appear to benefit the animal, as milk production and energy balance were not affected.

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CHAPTER 6

General Discussion

The Future Role of Ruminants in Nutrition of the Human Population

Ruminant products form an important component of the human diet and their inclusion in diets in developing countries is likely to increase over the coming decades, leading to an increase in the size of the global population of ruminants and its environmental impact (Steinfeld et al., 2006). Livestock, and ruminants in particular, have been identified as major contributors to the recent increase in greenhouse gas concentrations in the atmosphere (Steinfeld et al., 2006). When ruminant products are compared to other livestock products, e.g. pig or poultry meat, they consistently have a higher production of greenhouse gases per kg of animal protein (Figure 6.1; Williams et al., 2008). The production of greenhouse gases for ruminant products mainly originates from the production of enteric methane, the higher feed conversion ratio and the low number of progeny for ruminants (de Vries and de Boer, 2010).

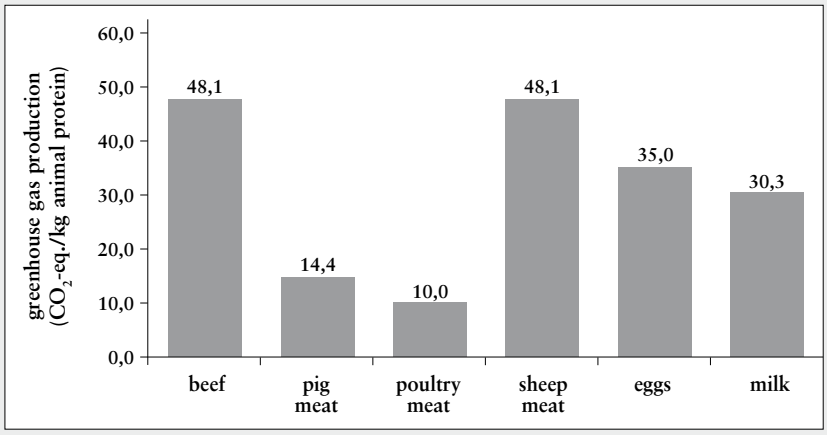

Figure 6.1

Greenhouse gas production (CO_2-eq/kg protein) for the main livestock products in the United Kingdom. Data were recalculated from Williams et al (2008), assuming protein contents of 27% for meat, 12% for eggs and 3.3% for milk.

From these data, a simple solution to the problem appears to lie in the shift of consumption of ruminant products to meat from non-ruminant species or vegetarian diets. However, the discussion about the environmental impact of livestock has been skewed by a narrow focus on the amount of greenhouse gases emitted per unit of product, rather than on the original goal of domesticated livestock: food production for the human population.

Ruminants have the unique capability of transforming non-edible feed for human consumption into valuable edible food and are important processors of roughages and food by-products (Gill et al., 2010, Wilkinson, 2011). With the increasing size of the human population, the competition between feed and food is likely to increase over the

years to come and the ability to convert protein unavailable to humans to human-edible protein will gain importance (Wilkinson, 2011). Figure 6.2 displays the amount of human edible protein that is required to produce 1 kg of animal protein in different animal production systems in the United Kingdom (Wilkinson, 2011), the USA and South-Korea (Gill et al., 2010).

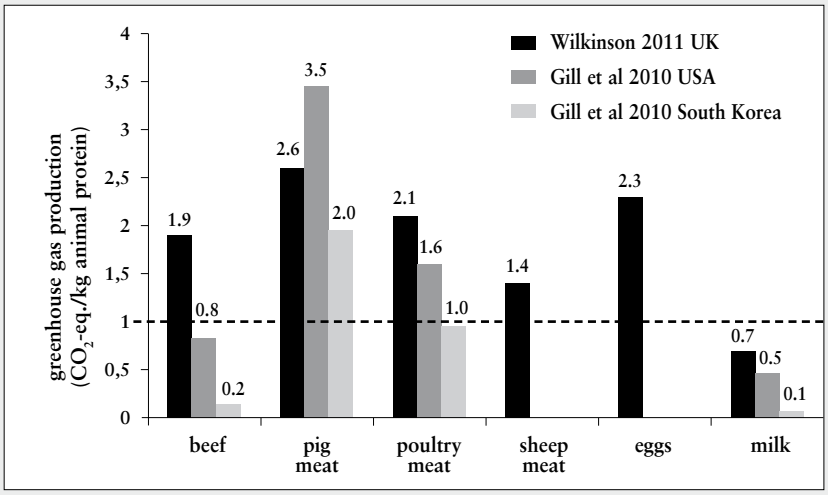

Figure 6.2

Use of human edible protein to produce animal protein for different production systems in the United Kingdom, USA and South-Korea. Values for beef and sheep are averages from the original publication of Wilkinson (2011). The dotted line represents the situation at which 1 kg of human-edible protein is used to produce 1 kg of animal protein.

When more human-edible protein is required than the actual amount of animal protein that is produced in animal systems, one might argue that it would be more efficient to directly consume the human-edible protein that is now fed to animals, i.e. consume plant rather than animal protein. With the increasing food demand as a consequence of the growing human population, the practice of feeding human-edible protein to animals will become more disputable, if it does not lead to a net increase of the availability of protein for human consumption.

From figure 6.2, it is clear that ruminant animals require less human edible protein to produce 1 kg of human-edible protein than monogastric animals. However, the amount of human-edible protein required depends on the type of diet that is fed to the animals. For instance, milk production is a net producer of human edible protein in all systems, but its efficiency in producing human-edible protein increases as the proportion of non human-edible roughage in the diet increases (South-Korea vs. UK data). This potential of ruminants should be further exploited by increasing the inclusion of roughages and

by-products that are of no value for human food into the ruminant diet (Wilkinson, 2011). Improvement of knowledge on how to most effectively feed products not edible by humans to ruminants will become an important topic to ruminant nutritionists in the coming decades.

Although ruminants will be able to play an important role in the future global food supply, this does not solve the issue of the increasing output of greenhouse gases as the population of livestock continues to grow and research to specifically reduce methane emissions from livestock remains necessary.

Relationship Between In Vitro and In Vivo Methane Reduction Studies

One of the aims of this thesis was to evaluate the in vivo effectiveness of dietary additives that had previously been proven to lower methanogenesis in vitro. Most of the products evaluated in the in vivo experiments described in Chapters 2-5 had been proven to reduce methane production in vitro, but failed to show significant mitigation in methane production in the in vivo studies (Chapter 3).

The relationship between methane production in vitro and in vivo has been argued to be poor before (Flachowsky and Lebzien, 2009). Several factors could be responsible for the low correlation between effectiveness in methane mitigation between in vitro and in vivo experiments, as will be discussed below. In this discussion, it is assumed that the additives tested are fed at the same inclusion level (g/kg DM substrate or feed) in the in vitro and in vivo experiments.

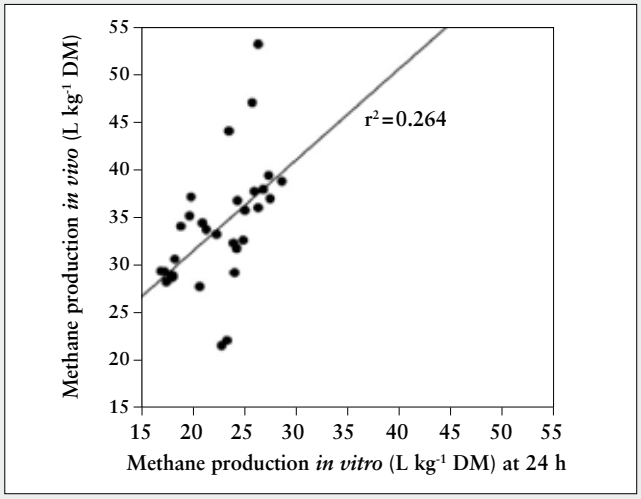

Figure 6.3

Relationship between methane produced in vivo and in vitro for a range of diets (Moss and Givens, (1997); cited in Flachowsky and Lebzien, (2009))

Differences in conditions between in vitro and in vivo experiments

The capacity of dietary additives to lower methane emissions is often initially evaluated with in vitro systems. In these systems, additives are added to a medium containing diluted, buffered rumen fluid maintained at body temperature to mimic in vivo rumen conditions. Rumen fluid is commonly obtained from rumen fistulated donor cows receiving a diet not containing the dietary additives (Lila et al., 2003, Goel et al., 2009, Holtshausen et al., 2009). The in vitro incubations generally last 1 or 2 d (batch culture systems; Patra et al., (2006), Kamel et al., (2008)) to 1 or more weeks (continuous culture systems; Busquet et al., (2005)).

In the in vitro systems, rumen fluid is diluted by a buffer in order to maintain pH throughout the incubation period. This dilution does, however, also mean that the bacterial density introduced from the rumen fluid is diluted. Consequently, the relative concentration of the test additive to the bacterial concentration increases by as much as dilution factor of the rumen fluid. Rumen fluid is typically diluted 2-4 times for in vitro studies (Asanuma et al., 1999, Goel et al., 2009). This could make the medium much more sensitive to perturbations from outside (e.g. the addition of methane lowering feed additives) relative to the original rumen fluid from the donor cow and observed in vitro effects may be more intense than those observed in the animal.

The donor cows are generally not fed the additive that is tested in the in vitro system. Thus, the microbial population in the donor rumen fluid is completely unadapted to the dietary additive. Upon introduction of the additive, the initial reduction in methane production may be substantial. Because the in vitro experiments are generally short-term, no adaptation to the dietary additive is likely to occur. In the animal, adaptation to dietary additives can occur after prolonged exposure to the additive (Guan et al., 2006). The effect of dietary additives has been demonstrated to be smaller, when the donor animals have already been adapted to the dietary additives (Domescik and Martin, 1997). The lack of adaptation in short-term in vitro incubations may lead to larger methane mitigation in vitro compared to the in vivo situation.

Rumen contents are subject to continuous dilution in the animal, as rumen fluid and digesta pass to the omasum, abomasum and subsequently to the lower intestinal tract. Consequently, dietary additive concentrations are diluted to a similar extent as the rumen contents. In vitro batch culture systems have no outflow of rumen fluid and consequently, the concentrations of dietary additives will remain at a high level when compared to the in vivo situation. In continuous flow in vitro systems, the outflow is simulated and these systems may more accurately approximate the potential of methane reducing feed additives.

In conclusion, larger methane lowering responses in vitro can be expected when the same level of additive (g/kg feed or substrate) is introduced in in vitro and in vivo experiments. This may mainly be due to a higher concentration of the additive in in vitro systems relative to the bacterial density, caused by rumen fluid dilution in vitro and rumen fluid outflow in vivo. Also, the rumen microbes may not have sufficient time to adapt to the dietary additives in vitro.

Dietary fat and specific fatty acids to reduce enteric methane production

In Chapters 2 and 3, attention has been paid to the capacity of specific fatty acids to lower methanogenesis. Fat addition to ruminant diets is one of the most frequently proposed strategies to reduce enteric methane emissions (Giger-Reverdin et al., 2003, Beauchemin et al., 2008, Eugene et al., 2008, Martin et al., 2010). The use of fat in ruminant diets has been demonstrated to reduce methane emissions by 2.2-5.6% for each additional 1% of crude fat added to the diet. The lowering of methane production by dietary fat addition is thought to work through one of the following mechanisms (Martin et al., 2010):

- Dietary fat is not fermented and thus dilutes the fermentable organic matter in the rumen.
- Specific fatty acids, medium chain fatty acids in particular, negatively affect the rumen methanogens.
- Specific fatty acids, unsaturated fatty acids in particular, have a negative effect on the cellulolytic bacteria and protozoa.
- Biohydrogenation of unsaturated fatty acids reduces the available hydrogen in the rumen and thus decreases methanogenesis.

Based on stoichiometrical principles, ruminal biohydrogenation of unsaturated fatty acids has been shown to have only a minor impact (2-3%) on methanogenesis at practical feeding levels of dietary fat (Martin et al., 2010).

In most studies published to date, the effect of dietary fat supplementation is compared with a diet not supplemented with fat. This experimental design often leads to reductions in feed intake and, as a consequence, observed methane reductions in these experiments are unrealistically large. Moreover, very high fat inclusion rates (>6.5% of DM) are often employed in these studies that negatively affect rumen fermentation and consequently lower methanogenesis (e.g. Martin et al., 2008). To circumvent these problems, in this thesis, we evaluated the effects of dietary fatty acids against isolipidic control diets, and feed intake was restricted to avoid negative impacts of the fat sources used on feed intake. In this way, the methane reducing potential of different individual fatty acids could be evaluated. We have used a maximum inclusion level of dietary fat of 6.5% of DM to avoid digestive disorders (NRC, 2001).

The experiment described in Chapter 2 was the only one in which we found a significant reduction in methane production that was most likely related to the intake of specific fatty acids. This reduction, however, was far lower than reported previously for these fatty acids (Machmüller, 2006, Martin et al., 2008). In experiment 2, described in Chapter 3, we were not able to detect any effects of the addition of C8:0 and C10:0 or extruded linseed in the diet on methane production. The diets in experiment 1 and 2 were fairly similar in composition, except for the crude fat content (3.3% for control treatment in experiment 1 and 5.8% for control treatment in experiment 2). There was a considerable difference in methane production between these two experiments (450 g CH_4/d for control treatment in experiment 1 and 371 CH_4/d for control treatment in experiment 2). This difference in methane production between experiments may at least in part have been caused by the different dietary fat concentrations between both experiments.

From previously published research it appears that elevation of dietary fat levels in ruminant diets may be a suitable way of lowering methane production. However, results presented in this thesis indicate that the fatty acid pattern of the added fat is of less importance than previously thought. This is confirmed by recent meta-analysis on the effects of fat addition on methanogenesis, in which no specific effects of fatty acid pattern of the added fat on methanogenesis were observed (Grainger and Beauchemin, 2011), although fat addition in general reduced methane emissions by 1 g methane/kg dry matter intake for each additional % of added dietary fat in cattle. The long-term persistency of elevated fat concentrations in the diet on methane emissions should be further evaluated.

Differences in the efficacy of dietary nitrate to reduce methane emissions

In the work described in this thesis, dietary nitrate was proven to be effective in lowering methane emissions in sheep and dairy cows (Chapters 4 and 5). Dietary nitrate was hypothesized to act as a hydrogen sink in the rumen, outcompeting methanogenesis for reducing equivalents. Based on the stoichiometry of the conversion of nitrate into ammonia, the hydrogen consuming potential of nitrate can be calculated. Because hydrogen used in the reduction of nitrate to ammonia can no longer be used for methanogenesis, the theoretical methane mitigation potential for nitrate is 25.8 g of CH_4/100 g of NO_3.

In the experiment described in Chapter 4, 89% of the theoretical methane reduction potential of nitrate was achieved when nitrate was fed to sheep (26 g nitrate/kg DM). When nitrate was fed to dairy cows (Chapter 5; 21 g nitrate/kg DM), only 59% of the theoretical methane mitigation potential was reached. Because Nolan et al. (2010) observed an efficiency of 78%, when nitrate was fed to sheep (24 g nitrate/kg DM), it appeared that the difference in efficacy of nitrate in methane mitigation might be related to species differences in feed intake level. However, in a later study the efficiency of nitrate in methane reduction, estimated using the SF_6-technique, was observed to be 88% in growing bulls (22 g nitrate/kg DM; Hulshof et al., 2011), weakening the argument that the efficacy of nitrate in methane mitigation would be related to species.

In later, unpublished work from our group, we have fed increasing levels of nitrate (0, 0.6, 1.1, 1.7, 2.2 and 2.7 g nitrate/kg DM) to growing steers (average body weight 337 kg), to assess the dose-response effect of incremental nitrate intake on methane emissions measured in respiration chambers. Dietary nitrate linearly reduced methane emissions with increasing dose, but the efficacy of nitrate in methane mitigation decreased with increasing dose (100%, 100%, 104%, 74%, 80% and 66% for the respective doses). This has led us to believe that the efficacy of nitrate in methane mitigation may be dose-related.

Although the dosages of nitrate per kg DM were similar among the published experiments, the level of feed intake, and thereby the actual nitrate intake, was quite different in the different experiments. To allow inter-species comparison, the exposure of animals in various studies to nitrate was expressed in g of nitrate per kg of metabolic weight per day.

In figure 6.4, results of all available studies to date employing dietary nitrate to reduce methane emissions are displayed. From this analysis, it appears that the effectiveness of dietary nitrate for reducing methane emissions decreases with increasing nitrate intake. The decreased efficacy at higher inclusion rates might be related to an insufficient capability of the rumen microbes to reduce dietary nitrate at high inclusion levels, thereby reducing the amount of hydrogen that is used in the reduction process.

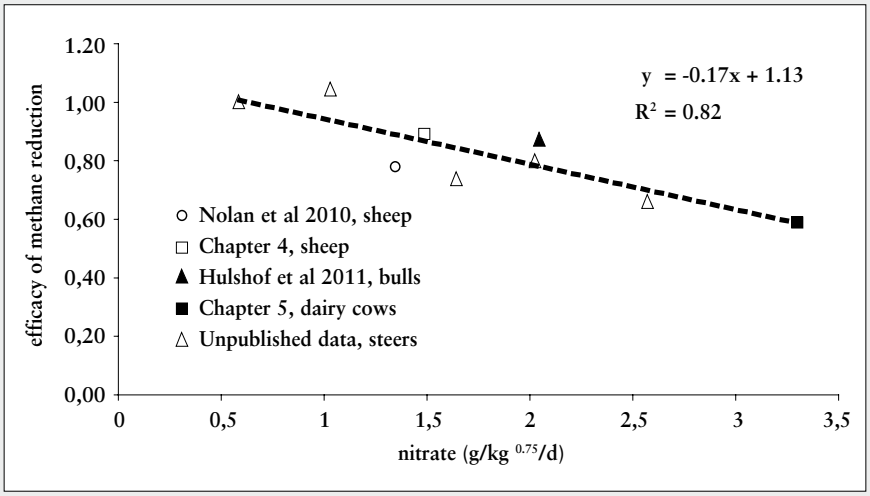

Figure 6.4

Efficiency of methane reduction (observed methane reduction / theoretical maximum methane reduction) with increasing doses of nitrate fed to various species of ruminant animals.

This hypothesis finds further support in the occurrence of elevated blood methemoglobin levels in the nitrate treatment of the study described in Chapter 5 (4.0% of Hb) and on the three highest levels of nitrate in the unpublished study (2.9, 5.1 and 11.6% of Hb, respectively), while in the other studies no elevated MetHb-levels were detected or not measured (Hulshof et al., 2011). This methemoglobin originates from the incomplete reduction of nitrate to ammonia. During this incomplete reduction, nitrite is formed, which is subsequently absorbed into the blood and forms methemoglobin (Bruning-Fann and Kaneene, 1993). Apparently there is a ceiling of approximately 1.0-1.5 g nitrate/kg $BW^{0.75}$/d, above which the rumen microbes are no longer capable of reducing all nitrate consumed, even if an adaptation period to dietary nitrate is used. The graph illustrates a linear decrease with increasing intake, not a ceiling-type of response.

Long-term persistency of methane reduction through dietary additives

In Chapter 5, dietary nitrate was demonstrated to persistently reduce enteric methane production over a period of 89-107 d. Persistency of the methane mitigating effect of dietary additives is an absolute requirement for the additive to be successful. To date, relatively little research has been conducted to investigate long-term effectiveness of methane mitigation strategies, quite likely because it is very time and labor consuming.

Monensin and lasolacid, ionophores that shift the rumen VFA pattern from acetate to propionate, are compounds that have probably been evaluated most for their persistency in methane mitigation. Guan et al. (2006) observed an initial 27% reduction in methane production during the first 4 wk of feeding ionophores, but this effect diminished and eventually disappeared over the 16-wk trial period. Similar results were obtained earlier by Johnson and Johnson (1995). Monensin was further evaluated for its persistency in a 6-month experiment by Odongo et al. (2007). Methane was depressed by 7% and this effect persisted over the entire trial period. The persistency of methane mitigation by ionophores may be related to the diet composition, as the methane mitigating effect lasted longer with lower concentrate inclusion levels in the diet (Guan et al., 2006).

Woodward et al. (2006) did not observe significant effects on methane production, when 300 g of oil/d (a mixture of linseed and fish oil) was fed to dairy cows after feeding the oils for 12 weeks. No methane measurements were done in an earlier stage of the experiment, making it difficult to disentangle potential initial and adaptive processes.

Results from previous long-term studies indicate that persistency of methane mitigating effects of dietary additives may be affected by diet type. Our study with dietary nitrate is the first to show a persistent (> 3 months) methane reduction with this additive on maize silage based diets. The experiment should be replicated with different diet types to assess if the persistency of the methane mitigation is diet-dependent. Nitrate may be less effective in diets with low initial methane production, i.e. diets containing a high proportion of concentrates. In these diets, there will be a low excess of hydrogen in the rumen, leaving also less reducing equivalents for nitrate reduction. The use of nitrate in ruminant diets is further limited by its high N content; inclusion of nitrate in diets that already contain a sufficient level of crude protein could lead to the excretion of excess N by the cow, with the potential of increased nitrous oxide emissions. This could negate the benefits obtained in methane mitigation.

Enteric Methane Production as a Loss of Energy to the Host Animal

Research to reduce enteric methane emissions from ruminants has evolved to become a large research area in ruminant nutrition. Reducing methane emissions from ruminants has already had the interest from the scientific community for several decades. The initial interest in the 1960's and 70's in this subject was to reduce the loss of dietary energy that methane represents and in this way enhance productivity or feed efficiency of the animal (Blaxter and Czerkawski, 1966). In work presented in this thesis, lactating dairy cows typically lost 5.7 to 7.4% of gross energy intake (GEI) as methane (Chapters 2, 3 and 5). Reducing methane emissions could therefore improve the animals' energy status by preventing this loss of energy (Czerkawski and Breckenridge, 1975).

In more recent decades, the interest in reducing methane emissions has shifted towards a reduction in the amount of greenhouse gas emissions produced by ruminants (Martin et al., 2010). Methane is a powerful greenhouse gas (23-25 CO_2-equivalents; Forster et al., 2007), and its production by ruminants significantly increases the amount of total greenhouse gases produced per unit of ruminant product when compared to other animal products from monogastric species (de Vries and de Boer, 2010). Although the major and very important objective in more recent research has shifted to lowering greenhouse gas production through methane mitigation, the prevention of loss of dietary energy is nearly always stated as a secondary objective to justify the research done. A reduction in the loss of methane energy is often automatically assumed to result in enhanced productivity of the animal of a magnitude similar to that of the size of the methane reduction.

However, results from energy balance and performance studies do not unanimously confirm that a reduction in methane production leads to enhanced productivity. Reductions in methane production as a consequence of dietary additives have been shown to improve energy retention in metabolism studies (Johnson, 1972, Johnson, 1974, Sawyer et al., 1974). Also, improvement in average daily gain has been demonstrated when chemical methane inhibitors were fed (Trei et al., 1972, Davies et al., 1982). The most consistent effect, however, is a reduction in feed intake with similar average daily gain, resulting in a lower feed: gain ratio (Trei et al., 1971, Cole and McCroskey, 1975, McCrabb et al., 1997).

The performance studies, in which positive effects of lowering methane on average daily gain or feed to gain ratio were observed, generally show lower effects than would be expected based on the extent of methane mitigation. Besides, there are also studies in which methane mitigation did not lead to improved animal performance (Sawyer et al., 1974) and sometimes negatively affected animal performance (Martin et al., 2008). In the experiments with dairy cows, described in Chapters 2 and 5 of this thesis, modest methane reductions of 10-16% were achieved, with total tract digestibility of the major nutrients remaining unaffected. However, we did not observe concomitant positive milk production responses nor increased body energy retention in our experiments.

In this discussion, we argue that it is not realistic to assume that the increase in metabolizable energy resulting from lowered methane emissions will be retained or used for milk production with a similar efficiency as metabolizable energy from feed. First, the methodology of measuring energy balance will be discussed, followed by a discussion of the role of hydrogen in measuring the energy balance. Last, the suitability of the Brouwer equation (Brouwer, 1965) for calculating heat production in experiments in which methane is specifically inhibited will be discussed.

Calculation of Energy Balances

In the calculation of energy balances for ruminants, the energy excreted with feces and urine, as well as energy losses in the form of combustible gases (methane and hydrogen), are subtracted from the gross energy ingested (GEI; Figure 6.5). The resulting energy is referred to as metabolizable energy (MEI), i.e. the energy that is potentially available to the animal for maintenance and production. Energy lost as heat, usually measured

from gaseous exchange using indirect calorimetry, is subsequently subtracted from MEI and the resulting energy is either deposited in body tissues, or excreted as milk. For lactating dairy cows, the amount of energy secreted in milk is further subtracted from the energy available and the resultant is the energy balance or energy retention (ER) of the ruminant.

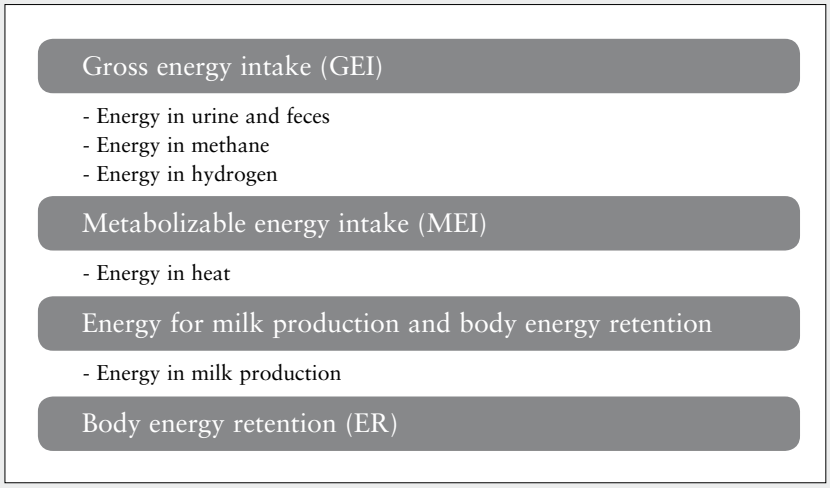

Figure 6.5

Schematic representation of calculation of energy balances in ruminant animals

The increased MEI from methane reduction is commonly assumed to be used for body energy gain or milk production with identical efficiency as other ME from feed. In our experiments with dairy cows (Chapters 2 and 5), we observed that approximately 42% of MEI was secreted as milk or retained within the body. If the increase in ME from reduced methane emissions in the experiment described in Chapter 5 would have been used with a similar efficiency for milk production as the other ME from feed (42%), this could have theoretically increased milk production by 0.5 kg or have increased body energy retention. Because neither was observed, possible energy losses to other processes, not picked up in the measurements, are discussed below.

Step-wise Analysis of Measurements in the Energy Balance

The energy lost in urine and feces is determined by quantitative collection of feces and urine and subsequent determination of its energy content through bomb calorimetry. This determination usually is quite accurate and is not likely to be subject to large errors.

The methodology of measuring methane energy output is very accurate in indirect respiration calorimetry (Verstegen et al., 1986). Hydrogen production, however, is often

not measured in experiments in which methane production is suppressed by dietary strategies (Chapter 2, Beauchemin et al., 2006, Holtshausen et al., 2009). Because hydrogen emissions may increase when methane is decreased by dietary strategies, not measuring hydrogen production may introduce a possible error in the determination of the energy balance (Johnson et al., 1972), as will be discussed below.

In indirect respiration calorimetry experiments, heat production is not determined directly, but calculated from the use of oxygen, and the production of carbon dioxide and methane inside the chamber, commonly applying the Brouwer equations (Brouwer, 1958, 1965). This method of calculating heat production is widely accepted, but may have some drawbacks for use in experiments in which methane production is specifically inhibited as outlined below.

The energy output in milk can also be accurately determined by quantitative collection of milk and subsequent determination of its energy content by bomb calorimetry.

The potential errors related to not measuring hydrogen production and the use of the Brouwer equation in these types of experiments is discussed below.

Shift from Enteric Methane to Hydrogen Emissions

Ruminants normally do not emit hydrogen, because ruminal methanogenesis very effectively captures the hydrogen that is released during rumen fermentation (Czerkawski, 1972). However, when methanogenesis is specifically inhibited, considerable hydrogen emissions can occur, leading to a shift from methane to hydrogen emissions (Trei et al., 1971, Johnson, 1972). According to Johnson et al. (1972) increased hydrogen emissions could account for up to 38% of the reduced energy emissions as methane. In more recent studies, enteric hydrogen production is often not measured and this could lead to an overestimation of the potential energy benefit of a reduction in methanogenesis. In the study described in Chapter 2, in which methane production was decreased by 10%, hydrogen production was not determined. In the experiment described in Chapter 5, hydrogen production was measured in the last out of 4 measuring periods over a 9-h period and it was observed that approximately 3.6% of the energetic benefit of methane reduction was offset by increased hydrogen production when nitrate was fed as an alternative hydrogen sink to methanogenesis.

The proportion of the shift from methane emissions to hydrogen emissions may depend on the method used for the inhibition of methanogenesis. In the experiment described in Chapter 5, nitrate was used as an alternative hydrogen sink to methanogenesis. Providing and alternative hydrogen sink likely captures a major portion of the hydrogen that would normally be lost through methane emissions or direct emissions of hydrogen. However, in studies in which methane was chemically inhibited, without providing an alternative hydrogen sink, up to 38% of the energy spared from methane reduction, was lost through hydrogen emissions (Johnson et al., 1972).

Hydrogen not only plays a role in the energy balance of the ruminant, but also has indirect effects on the accumulation of greenhouse gases in the atmosphere. In the

atmosphere, hydrogen reduces the levels of OH-radicals, thereby increasing the lifespan of some direct greenhouse gases like methane (Forster et al., 2007). When the indirect effects of hydrogen are taken into account, the global warming potential of hydrogen has been estimated at 5.8 CO_2-eq./kg on a 100-yr time scale (Derwent et al., 2001).

Although our measurements of hydrogen production (chapter 5) did not reveal its quantitative importance in the energy balance, the quantitative importance likely depends on the size of the reduction of methane production and also the method used in the mitigation strategy. In addition, hydrogen adds to the greenhouse gas emissions directly. Therefore, we suggest to quantify hydrogen production in future studies of methane mitigation strategies.

Conversion of Spared Methane Energy into Heat

Heat production in indirect respiration calorimetry is calculated from the amount of oxygen used and the amount of carbon dioxide produced by the animals inside the chamber (Brouwer, 1958, 1965). The calculation of heat production is based on the fact that carbohydrates, fat and protein require a fixed volume of oxygen to be fully oxidized and that fixed amounts of carbon dioxide and heat are produced during this oxidation. Consequently, there is a direct, proportional relationship between the volumes of oxygen consumed, carbon dioxide produced and the heat produced during oxidation (equation 1; Brouwer, 1965). For this equation it is assumed that carbohydrates, fat and protein are fully oxidized to carbon dioxide and water. Heat production is corrected for nitrogen that is excreted in urine.

$$\mathbf{T = 16.175 \times O_2 + 5.021 \times CO_2 - 5.987 \times N} \qquad \text{(equation 1)}$$

In which:
T = Heat production (kJ/d)
O_2 = Oxygen consumption (L/d)
CO_2 = Carbon dioxide production (L/d)
N = Nitrogen excreted in urine by the animal (g/d)

Equation 1 is only valid for animals not producing methane. In ruminants, carbohydrates and protein can be converted into VFA by anaerobic fermentation. VFA production represents incomplete oxidation of protein and carbohydrates in the rumen. During fermentation, carbon dioxide is produced, which is included in the Brouwer equation to calculate heat production. The VFA formed in the rumen can be absorbed and further oxidized in the metabolism of the animal, using oxygen and producing carbon dioxide. The VFA metabolism in the tissues of the animal satisfies the Brouwer equation (Equation 1), as it is an oxidative process utilizing oxygen and producing carbon dioxide during its metabolism.

During the fermentation process, part of the energy from the feed is lost as methane. The methane emitted by the animal has not been oxidized to carbon dioxide and thus does not satisfy the assumptions made for equation 1. Brouwer (1958) developed a methane correction factor to account for the reduced heat production when methane is

produced. He considered the full, aerobic oxidation of methane (equation 2), which uses 2 L of oxygen and produces 1 L of carbon dioxide. It was assumed that if ruminants would not produce methane, they would oxidize the methane, thereby increasing oxygen consumption and carbon dioxide production by 2 L and 1 L, respectively, for each L of methane not produced.

$$CH_4 + 2O_2 \rightarrow CO_2 + 2\ H_2O \qquad \text{(equation 2)}$$

In the methane-corrected equation (equation 3), the volumes of oxygen and carbon dioxide that would have been produced if the methane had been oxidized within the animal are added to the volumes shown in equation 1. Subsequently, the energy value of methane (39.5 MJ/L) is subtracted from this. The combination of this yields a methane correction factor of -2.167 kJ of heat/L of methane produced.

$$T = 16.175 \times O_2 + 5.021 \times CO_2 - 5.987 \times N - 2.167 \times CH_4 \qquad \text{(equation 3)}$$

In which CH_4 = methane production in L/d.

Indirectly, the heat produced in the process of methanogenesis is accounted for in this equation: If methane production is reduced by 1 L by selective inhibition of methanogenesis, CO_2 production will go up by 1 L as a consequence of the stoichiometry of methanogenesis (equation 4):

$$CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O \qquad \text{(equation 4)}$$

The negative term for CH_4 in equation 3 implies that for each liter of methane reduction, heat production will be increased by 2.167 kJ/d. The positive term for CO_2 results in a further heat production of 5.021 kJ/d with every L of decrease in methane production, because CO_2-production stoichiometrically increases by 1 L. This increase would be part of the measured CO_2 production. One liter of methane contains 39.5 kJ/L. Consequently, 18% (= 2.167 + 5.021 / 39.5) of the energy spared from methane reduction is always lost as heat, and the complementary portion (82%) would be available for energy retention or milk production. This energetic benefit is used in the current approach to calculate energy balances and the energy spared through methane reduction is assumed to be available to the animal for milk production or body energy retention with an efficiency of 82%.

Contribution of Methane to Calculated Heat Production

The negative term for methane in equation 3 leads to increased heat production when methane is inhibited. However, the contribution of the methane term to the calculated heat production is very small. For a dairy cow, using 5500 L oxygen and producing 6000 L of carbon dioxide and 500 L of methane/d, less than 1 % of the calculated heat production results from the methane term in the equation (*Table 6.1*). Consequently, erroneous assumptions in the methane term will probably not be noticed in the calculated heat production and it has even been proposed to exclude the methane term from the Brouwer equation, because of its low effect on the resulting calculated heat production (McLean, 1986). However, in studies in which methane is lowered, such a wrongful

assumption could have severe consequences on the calculated efficiency with which the energy saved from methane production can be utilized (see also page 110). If methane would be fully absent in this example, heat production would only increase by 1%.

Table 6.1

Typical consumption of oxygen and production of carbon dioxide and methane for a lactating dairy cow and its contribution to the calculated heat production according to equation 3; Brouwer (1965)

	Volume used/produced (L/d)	Calculated heat production (MJ/d)	Contribution to heat production (%)
Gas type			
Oxygen	5,500	89.0	74
Carbon Dioxide	6,000	30.1	25
Methane	500	1.1	1
Total	12,000	120.2	100

Consequences of Methane Inhibition on Rumen Fermentation and Heat Production

In the assumptions made for the Brouwer equation, methane is assumed to be oxidized if it is not exhaled by the animal. Methane oxidation, however, is a process that is of little significance in the rumen (Kajikawa et al., 2003). Methanogenesis in the rumen serves to remove metabolic hydrogen, and prevention of methanogenesis generally leads to accumulation of hydrogen in the rumen. This accumulated hydrogen can be breathed out by the animal or incorporated in reduced end product (e.g. propionate, ammonia). When hydrogen accumulates in the rumen, a shift from acetate (equation 5) and butyrate (equation 7) to propionate production (equation 6) is commonly observed (Ellis et al., 2008).

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2C_2H_3O_2^- + 2CO_2 + 4H_2 + 2H^+ \quad \text{(equation 5)}$$

$$C_6H_{12}O_6 + 2H_2 \rightarrow 2C_3H_5O_2^- + 2H^+ + 2H_2O \quad \text{(equation 6)}$$

$$C_6H_{12}O_6 \rightarrow C_4H_7O_2^- + H^+ + 2H_2 + 2CO_2 \quad \text{(equation 7)}$$

During any reaction that occurs spontaneously in the rumen, energy is released in the form of heat. The total energy released during a reaction is referred to as Gibbs free energy (ΔG). Only reactions with a negative value for Gibbs free energy (i.e. processes that release energy in the form of heat) can occur spontaneously (Ungerfeld and Kohn, 2006). The Gibbs free energy values for acetate, propionate, butyrate and methane production under typical rumen conditions are given in table 6.2. In this table, the Gibbs free energy value for nitrate reduction to ammonia is also shown.

The formation of methane from carbon dioxide and hydrogen is a relatively simple process. Due to its simplicity, relatively little heat is produced during its formation. The formation of the more complex reduced end products (e.g. propionate from glucose, ammonia from nitrate) is a process, during which more heat is produced (*Table 6.2*). When methane production is inhibited and hydrogen will be incorporated into propionate, it is likely that a larger proportion of the energy contained in hydrogen is lost in heat production.

Table 6.2

Gibbs free energy values for fermentation of glucose to acetate, propionate and butyrate and Gibbs free energy values for methanogenesis and nitrate reduction under typical rumen conditions (Kohn and Boston, 2000)

	ΔG reaction (kJ/mol)	ΔG / mol H_2 used (kJ/mol)
Reaction		
Glucose to Acetate	-318	-
Glucose to Propionate	-322	-161
Glucose to Butyrate	-312	-
Methanogenesis	-68	-17
Reduction of nitrate to ammonia	-501	-125

Ruminal hydrogen contains 284 kJ/mol of H_2 (Afeefy et al., 2011). If this hydrogen would be converted into methane, only 6% of the energy would be converted into free energy available for ATP formation or heat production (= 17/284). Consequently, most of the energy first contained in hydrogen is emitted from the animal in the form of methane.

If methane production is inhibited and hydrogen is incorporated into propionate, 57% (= 161/284) of the hydrogen energy is converted into free energy available for ATP formation and heat generation. When nitrate is used as an alternative electron acceptor, 44% (= 125/284) of the energy contained in the hydrogen is lost as free energy. These data indicate that a larger part of the energy contained in hydrogen is lost as heat, when hydrogen is used in alternative processes to methanogenesis (propionogenesis and nitrate reduction).

This discussion indicates that the consequences of a reduction in methane emission are misrepresented in the equation of Brouwer (1965). In this equation, the spared methane from a lowering of methane production is assumed to be oxidized by the animal. In reality, a reduction in methane production leads to the alternative use of hydrogen in the formation of other reduced end products like propionate or ammonia. When hydrogen is used for these processes, rather than methanogenesis, more energy from hydrogen is lost for the formation of the reduced end products. This is a likely explanation for the lower than expected effects on animal energy status when methane production is inhibited. It also confirms earlier work by Czerkawski (1986), in which it was observed

that the Brouwer equation is only valid when the VFA-profile remains constant. The Brouwer equation can be used in energy balance studies with ruminants, because the methane term contributes little error to the calculated heat production. However, when methane production is specifically inhibited, the lack of accurate representation of heat production from the alternative use of hydrogen in reduced end products may result in an overestimation of the energy available to the animal.

Conclusions and Recommendations

In the work described in this thesis, most of the dietary additives, that had been proven effective in vitro, were not effective in reducing methane production in vivo (Chapter 3). Several hypotheses for this discrepancy in results have been formulated (Chapter 6). It is recommended to always follow up in vitro experiments by in vivo experiments to ensure in vivo efficacy. Successful methane mitigations by dietary additives tested in in vitro studies should be communicated with care.

The addition of fat to ruminant diets appeared effective in reducing methane emissions (Chapter 3). This reduction in methanogenesis mainly originates from an absolute increase of the level of fat in the diet; the fatty acid pattern of the added fat had only minor (-10%, Chapter 2) or no effects (Chapter 3) on methanogenesis in limit-fed isolipidic diets. The persistency of the methane-lowering effect of fat addition has not been investigated thoroughly and this subject merits further research.

Dietary nitrate and sulfate effectively lower enteric methane production from ruminants (Chapter 4) and the methane-lowering effect of nitrate is persistent (>3 months) in dairy cows fed a corn silage-based diet (Chapter 5). The persistency of this effect should be further investigated in different diet types. Although nitrate was effective in lowering methane, its effectiveness decreased with increasing dose (expressed in g/kg $BW^{0.75}$ per day; Chapter 6) and it was found to be most effective in the range of 1.0 to 1.5 g nitrate/kg $BW^{0.75}$ per day. Higher doses also increase the methemoglobin levels in blood. Further research should explore the factors limiting nitrite reduction in the rumen to be able to remove this barrier and allow nitrate to be used more effectively in methane mitigation.

In the studies, in which methane production was successfully decreased and the energy balances of the animals were determined (Chapters 2 and 5), no benefits of the decrease in methane production on milk production or energy retention were observed. Some possible explanations for this are given in Chapter 6. In future experiments, hydrogen emissions should be measured, because they may increase as methane is inhibited. This could partly offset the energetic benefit gained from the methane decrease. The Brouwer equation to calculate heat production should be employed with care in studies in which methane is selectively inhibited. This equation assumes that the methane not emitted by the animal is oxidized within the tissues. In reality, hydrogen not used for methanogenesis is used to form other reduced end products, a process during which more heat is produced than from methanogenesis. Use of the Brouwer equation in studies in which methane is specifically inhibited may result in an overestimation of the energy available to the animal.

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Summary / Samenvatting

Summary

Ruminants occupy a unique niche in the animal kingdom, because of their ability to convert structural cell wall components into forms of energy and protein that can be used by humans. In a symbiotic collaboration, microfora and-fauna in the ruminants' intestinal tract break down and ferment fibrous material to a form usable by the ruminant. This enables the ruminant to convert materials not edible for humans into available forms of protein and energy for humans. This property has led to the vast domestication of ruminants and their current global population is estimated at 3.6 billion. This population is anticipated to grow further, as the global human population, and its demand for animal products, continues to grow. The demand for ruminant products is expected to increase by more than 50% over the next two decades.

Anaerobic microbial fermentation taking place in the rumen is accompanied by the production of methane. Methane is a loss of energy to the animal as well as a greenhouse gas and for these reasons, many research projects have been initiated to reduce methane emissions from ruminants. From this research, dietary strategies have been developed to reduce methane emissions. However, many of these strategies have only been tested in laboratory conditions.

The first aim of this thesis was to investigate if strategies that were successful in reducing methane emissions in vitro would also be successful when applied in vivo. The second aim of the thesis was to assess if short-term (3 weeks) reductions in methane production in vivo would persist for a longer period of time (4 months). The third aim of the thesis was to evaluate if a lowering of methane production would benefit the animals' energy status.

In Chapter 2, two experiments are described with the aim to investigate if the addition of dietary additives can lower methane emissions from lactating dairy cows. A mixture of linseed oil, lauric acid, myristic acid and fumarate was fed to a group of lactating cows, whereas a group of cows on the control treatment received an isolipidic diet containing saturated fat. Methane production was lowered by 10% in the cows fed the dietary additives (362 g/d vs. 325 g/d). Although methane production was lowered by feeding the dietary additives, the energy balance of the cows was not significantly affected. Fat and protein corrected milk production was lower for the groups receiving the dietary additives. The results of this experiment show that enteric methane production can be lowered through dietary strategies. The results indicate also that previously reported reductions in methane production due to addition of these fats and oils appear to be largely caused by indirect methane decreases as a consequence of lower DMI and different fat contents between diets.

In Chapter 3, the effect of 5 individual dietary additives on methane production from dairy cows is evaluated in two experiments. The additives investigated were diallyl disulfide, yucca powder, calcium fumarate, an extruded linseed product and a mixture of capric and caprylic acid. These additives had all been demonstrated to have methane lowering potential in vitro. However, in the in vivo experiments, none of the additives lowered methane production. A large difference in methane production was observed between

both experiments (25.5 vs. 23.2 g of methane/kg of DM), which may have originated from the difference in fat contents of the diets used in the experiments. The addition of capric and caprylic acid improved fat digestibility and increased milk fat content. Energy balances were not affected by dietary treatments.

In the experiment described in Chapter 4, nitrate (2.6% of DM) and sulfate (2.6% of DM) were fed to growing lambs to assess the potential of these additives to lower methanogenesis. The addition of nitrate reduced methane emissions by 32%, whereas the addition of sulfate to the feed reduced methane emissions by 16%. When both products were fed, methane production was reduced by 47%. In this experiment, lambs were gradually introduced to the dietary additives over a period of 4 weeks and no adverse effects of feeding the additives were observed. The efficacy of nitrate and sulfate use in methane mitigation was calculated to be 89% and 67% of the stoichiometric potential, respectively. From this study, nitrate and sulfate appeared suitable additives for reducing methane emissions from ruminants.

The use of nitrate was further evaluated in an experiment with dairy cows (Chapter 5). The aim of this experiment was to evaluate the methane mitigating effects of feeding nitrate in dairy cows and also to assess if the reduction in methane production would be persistent. Methane production was reduced by 16% without any effects on diet digestibility and this effect persisted over the entire 89-d trial period. Milk production was not affected by nitrate feeding, but the milk protein concentration was lower in the cows fed nitrate. The energy balance was not affected by the dietary treatment. Methemoglobin levels in blood were elevated for cows fed nitrate, but peak levels were far below the levels considered to cause subclinical methemoglobinemia.

The correlation between methane reductions observed in vitro and in vivo is low and most of the strategies tested in this thesis resulted in no methane reduction in vivo, while their efficacy had previously been shown in vitro. This lack of correlation could result from several factors. In in vitro systems the rumen fluid is commonly diluted by addition of a buffer, which lowers the bacterial density and, consequently, increases the proportion of the additive relative to the bacterial density. Furthermore, donor cows from which the rumen fluid is obtained, usually do not receive the dietary additives and micro-organisms in rumen fluid are not adapted to the dietary additives. In vivo, adaptation may occur. Also, in vitro systems are often closed systems and fail to represent the outflow of additives and substrate as would be the case in the live animal.

Addition of dietary fat may be a suitable strategy to reduce enteric methane emissions from ruminants. This confirms results from earlier analyses. However, results of research in this thesis indicate that the fatty acid profile of the added fat source is of minor importance in lowering methane production.

The efficacy of nitrate in reducing enteric methane emissions decreases with increasing dose (g/kg $BW^{0.75}$ per day). The efficacy decreases above doses of 1.0 to 1.5 g nitrate/$kg^{0.75}$ per day. Above this dose, methemoglobin concentrations in blood are also elevated, possibly indicating inefficient nitrite reduction in the rumen when higher doses are introduced into the diet. Nitrate was observed to induce persistent methane reductions

in this study, but this effect should be confirmed in additional studies using different diets.

Methane production is a loss of dietary energy to the animal and a reduction in enteric methane production is often assumed to result in improved productivity or energy retention by the animal, provided that feed digestibility is not negatively affected. In two studies described in this thesis (Chapters 2 and 5), methane production was successfully lowered and feed digestibility was unaffected by the methane lowering strategy. However, no beneficial effects on milk production or energy retention were observed. Several potential factors explaining this lack of improvement in energy status are discussed in Chapter 6. Hydrogen production is often not measured in experiments in which methane reducing strategies are investigated. However, energy losses from hydrogen can be substantial when methane is inhibited and it is recommended to measure hydrogen production in future studies.

The assumptions made in the Brouwer equation, which is commonly used in the calculation of heat production, in indirect respiration calorimetry may not be valid in experiments where methane is specifically inhibited. The use of hydrogen in reduced end products as a consequence of methane inhibition may lead to more heat production than is commonly assumed. The increase in metabolizable energy gained from a methane reduction, may be used less efficiently than is commonly assumed.

In conclusion, many dietary strategies to reduce methane emissions from ruminants are effective in vitro, but fail to show a methane lowering effect in vivo. The addition of dietary fat appears an effective way of reducing methane emissions, although the fatty acid pattern of the added fat seems to be less important. Nitrate persistently reduces methane production. Despite methane reductions, no improvements in milk production or energy retention were observed in these studies.

Samenvatting

Herkauwers bezetten een unieke plaats in het dierenrijk doordat zij in staat zijn om structurele plantcelwanden om te zetten in bruikbare vormen van energie en eiwit. In een symbiotisch samenwerkingsverband wordt door de microflora en –fauna in het maagdarmkanaal van de herkauwer vezelachtig materiaal afgebroken, gefermenteerd en omgezet in een bruikbare vorm voor de herkauwer. Dit stelt de herkauwer in staat om niet-eetbare materialen voor mensen om te zetten in waardevolle vormen van eiwit en energie voor de mens. Deze eigenschap heeft tot de uitgebreide domesticatie van herkauwers geleid en de huidige wereldwijde populatie herkauwers wordt op 3,6 miljard dieren geschat. Het wordt voorzien dat de populatie verder zal groeien doordat de humane bevolking, en haar vraag naar dierlijke producten, blijft groeien. De vraag naar herkauwerproducten wordt verwacht met meer dan 50% toe te nemen in de komende twee decennia.

De anaerobe microbiele fermentatie die plaatsvindt in de pens gaat gepaard met de productie van methaan. Methaanproductie is zowel een verliespost van energie voor het dier als een broeikasgas en om deze redenen zijn vele onderzoeksprojecten geïnitieerd om de methaanuitstoot door herkauwers terug te dringen. Vanuit dit onderzoek zijn vele voedingsstrategiëen ontwikkeld om methaanemissies te reduceren. Echter, vele van deze strategieën zijn alleen onderzocht onder laboratoriumcondities.

Het eerste doel van dit proefschrift was te onderzoeken of strategiëen die in vitro succesvol waren in het reduceren van methaanemissies ook succesvol zouden zijn wanneer deze in vivo toegepast zouden worden. Het tweede doel van dit proefschrift was te beoordelen of kortdurende (3 weken) methaanverlagingen ook zouden voortduren over een langere periode (4 maanden). Het derde doel was te beoordelen of een verlaging van de methaanproductie de energiestatus van het dier ten goede zou komen.

In Hoofdstuk 2 zijn twee experimenten beschreven met het doel te onderzoeken of de toevoeging van voedingsadditieven methaanemissies van lacterende melkkoeien kunnen verlagen. Een mengsel van lijnzaadolie, laurinezuur, myristinezuur en fumaraat werd gevoerd aan een groep melkkoeien, terwijl een groep koeien op de controlebehandeling een rantsoen gevoerd kregen met eenzelfde vetgehalte uit verzadigd vet. De methaanproductie was 10% lager voor de koeien die de additieven gevoerd kregen (362 g/d vs. 325 g/d). Hoewel de methaanproductie door het voeren van de additieven verlaagd werd, werd de energiebalans van de koeien niet significant beinvloed. Melkproductie, gecorrigeerd voor vet en eiwit, was lager voor de groep die de additieven kreeg. De resultaten van het experiment tonen aan dat eerder gevonden methaanverlagingen door het toevoegen van deze vetten en oliën grotendeels veroorzaakt lijken te zijn door indirecte methaanverlagingen als gevolg van lagere voeropname en verschillende vetgehaltes tussen rantsoenen.

In hoofdstuk 3 zijn de effecten van vijf individuele additieven op methaanproductie van melkkoeien geëvalueerd in twee experimenten. De onderzochte additieven waren diallyl disulfide, yucca poeder, calcium fumaraat, een geëxtrudeerd lijnzaadproduct en een mengsel van caprinezuur en caprylzuur. Van deze additieven was het methaanverlagend

potentieel in vitro eerder aangetoond. Echter, in de in vivo experimenten verlaagde geen van de additieven de methaanproductie. Er werd een groot verschil in methaanproductie waargenomen tussen beide experimenten (25.5 vs. 23.2 methaan/kg DS), hetgeen veroorzaakt zou kunnen zijn door het verschil in vetgehalte van de in de experimenten gebruikte rantsoenen. De toevoeging van caprinezuur en caprylzuur verhoogde de vetverteerbaarheid en verhoogde het melkvetgehalte. De energiebalans werd niet beinvloed door de behandelingen.

In het experiment beschreven in Hoofdstuk 4 werden nitraat (2,6% van de DS) en sulfaat (2,6% van de DS) aan groeiende lammeren gevoerd om het potentieel van deze additieven om methaan te verlagen te beoordelen. De toevoeging van nitraat verlaagde methaanemissies met 32%, terwijl de toevoeging van sulfaat methaanemissies met 16% verlaagde. Wanneer beide producten gevoerd werden, werd de methaanproductie met 47% gereduceerd. In dit experiment werden de lammeren geleidelijk blootgesteld aan de additieven gedurende een periode van 4 weken en er werden geen negative effecten van het voeren van de additieven waargenomen. De efficiëntie waarmee nitraat en sulfaat werden benut voor methaanreductie werd berekend op respectievelijk 89% en 67% van het stoichiometrisch potentieel. Uit dit experiment bleken nitraat en sulfaat geschikte additieven om methaanemissies van herkauwers te verlagen.

Het gebruik van nitraat werd verder beoordeeld in een experiment met melkkoeien (Hoofdstuk 5). Het doel van dit experiment was het methaanverlagende effect van nitraat te evalueren bij melkkoeien en daarnaast te beoordelen of de verlaging van methaanproductie persistent zou zijn. Methaanproductie werd verlaagd met 16%, zonder effecten op rantsoenverteerbaarheid en dit effect duurde voort gedurende de gehele 89-daagse proefperiode. Melkproductie werd niet beïnvloed door het voeren van nitraat, maar het melkeiwitgehalte was lager voor de nitraat gevoerde koeien. De energiebalans werd niet beïnvloed door de behandeling. Methemoglobine-niveaus in bloed waren verhoogd voor de nitraat gevoerde koeien, maar de piekniveau's waren ver onder de niveau's die beschouwd worden als oorzaak voor subklinische methemoglobinemia.

De correlatie tussen methaanreducties gevonden in vitro en in vivo is laag en veel van de strategiëen onderzocht in dit proefschrift resulteerden niet in een methaanreductie in vivo, hoewel de doetreffendheid van de maatregelen eerder in vitro was aangetoond. Deze lage correlatie zou veroorzaakt kunnen worden door verschillende factoren. In in vitro systemen wordt pensvloeistof gewoonlijk verdund door de toevoeging van een buffer, hetgeen de bacteriele dichtheid verlaagt en, dientengevolge, de verhouding van de concentratie van het additief ten opzichte van de bacteriele dichtheid verhoogt. Bovendien ontvangen de donorkoeien, waarvan de pensvloeistof verkregen wordt, gewoonlijk niet de additivieven en micro-organismen in de pensvloeistof van de donorkoeien zijn niet geadapteerd aan het additief. In de in vivo situatie zou adaptatie voor kunnen komen. Daarnaast zijn in vitro systemen vaak gesloten systemen en wordt de uitstroom van substraat en additieven zoals dit in het levende dier gebeurt, niet nagebootst.

Het toevoegen van vet aan het rantsoen zou een geschikte strategie kunnen zijn om methaanemissies van herkauwers te reduceren. Dit bevestigt resultaten van eerdere analyses. Echter, resultaten in dit proefschrift wijzen erop dat het vetzuurprofiel van de

toegevoegde vetbron van gering belang is voor het verlagen van de methaanproductie.

De eficientie waarmee nitraat methaanproductie verlaagt wordt minder naarmate de dosering (g/kg lichaamsgewicht$^{0.75}$ per dag) toeneemt. De efficiëntie neemt af bij dosering boven 1,0 tot 1,5 g/kg lichaamsgewicht$^{0.75}$ per dag. Boven deze dosering nemen de methemoglobinegehaltes in bloed ook toe, hetgeen mogelijk een indicatie is van inefficiente nitrietreductie in de pens wanneer hogere dosering in het rantsoen worden geïntroduceerd. Nitraat induceerde een persistente methaanreductie, maar dit effect dient te worden bevestigd in verder onderzoek met verschillende rantsoenen.

Methaanproductie is een verliespost voor het dier en een verlaging van de methaanproductie wordt vaak verondersteld te resulteren in een verbeterde productie of energieretentie voor het dier, vooropgesteld dat de rantsoenverteerbaarheid niet negatief beinvloed wordt. In twee experimenten beschreven in dit proefschrift (Hoofdstukken 2 en 5), werd de methaanproductie succesvol verlaagd en werd de rantsoenverteerbaarheid niet beinvloed door de methaanverlagende strategie. Er werden echter geen positieve effecten op melkproductie of energieretentie waargenomen. Verscheidene mogelijke oorzaken die dit gebrek aan verbetering zouden kunnen verklaren zijn bediscussieerd in Hoofdstuk 6. De productie van waterstof wordt vaak niet bepaald in experimenten waarin methaan wordt verlaagd. De energieverliezen als gevog van waterstofverliezen kunnen echter aanzienlijk zijn, wanneer methaanproductie geremd wordt en het wordt aanbevolen waterstofproductie in toekomstige studies te meten.

De veronderstellingen die gebruikt worden in de Brouwer-vergelijking, die gewoonlijk gebruikt wordt voor de berekening van warmteproductie in indirecte respiratie calorimetrie, zouden niet geldig kunnen zijn in experimenten waarin methaan specifiek geremd wordt. De verandering in vluchtig vetzuurprofiel als gevolg van methaanremming zou kunnen leiden tot meer warmteproductie dan gewoonlijk wordt aangenomen. De toename in metaboliseerbare energie als gevolg van methaanreductie zou minder efficient gebruikt kunnen worden dan gebruikelijk wordt aangenomen.

Concluderend kan worden gesteld dat vele methaanverlagende strategiëen effectief zijn in vitro, maar geen effect laten zien in de in vivo situatie. De toevoeging van vet aan het rantsoen lijkt een effectieve manier om methaanemissies te verlagen, hoewel het vetzuurpatroon van het toegevoegde vet van gering belang lijkt te zijn. Nitraat verlaagt methaanproductie op persistente wijze. Ondanks de waargenomen methaanverlagingen werden geen verbeteringen in melkproductie of energieretentie waargenomen in deze experimenten.

Curriculum Vitae

Curriculum Vitae

Sander van Zijderveld werd op 8 oktober 1977 geboren te Dordrecht. In 1998 behaalde hij zijn VWO-diploma aan het Develsteincollege te Zwijndrecht. In hetzelfde jaar begon hij aan een studie Veehouderij aan de Hogere Agrarische School te Delft. Deze opleiding rondde hij in 2002 af in de afstudeerrichting Diergezondheidszorg. In 2002 vond hij zijn eerste baan als assistent-onderzoeker Rundveevoeding bij Schothorst Feed Research te Lelystad. In 2005 werd hij Onderzoeker Rundveevoeding bij datzelfde instituut. In 2007 ging hij als Onderzoeker Rundveevoeding werken voor Provimi te Rotterdam. Als onderdeel van deze baan volgde hij een promotietraject bij Wageningen Universiteit. Het onderzoek uit dit project is beschreven in dit proefschrift.

Publications

Refereed Scientific Publications

Van Zijderveld, S. M., W. J. J. Gerrits, J. A. Apajalahti, J. R. Newbold, J. Dijkstra, R. A. Leng, H. B. Perdok. 2010. Nitrate and sulfate: Effective alternative hydrogen sinks for mitigation of ruminal methane production in sheep. J. Dairy Sci. 93, 5856-5866.

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Contributions to Conferences and Symposia

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Training and supervision plan

Training and supervision plan

Name	Sander van Zijderveld
Group	Animal Nutrition Group
Daily supervisors	Jan Dijkstra and Walter Gerrits
Supervisor	Wouter Hendriks

The basic package	**year**	**credits**
WIAS Introduction Course	2007	1.5
Ethics and Philosophy of Animal Science	2008	1.5
Scientific Exposure		
International conferences		
American Dairy Science Association conference (Indianapolis 7-11 juli 2008)	2008	1.5
International Symposium on Ruminant Physiology (Clermont-Ferrand 6-9 sept. 2009)	2009	1.2
Non CO2 Carbon Greenhouse Gas conference (Wageningen, 30 Juni-3 juli 2009)	2009	1.2
Greenhouse Gases and Animal Agriculture Conference (Banff 3-8 oktober 2010)	2010	1.5
Presentations		
ADSA 2008 (oral presentation)	2008	1.0
ISREP 2009 (poster presentation)	2009	1.0
NCGG-5 (oral presentation)	2009	1.0
GGAA 2010 (poster presentation)	2010	1.0
In-Depth Studies		
Disciplinary and interdisciplinary courses		
WBS cursus rundveevoeding	2003	0.6
Course “Advances in Feed Evaluation Science”	2011	1.5
Advanced statistics courses		
Course “Design of experiments”	2010	1.0
Course “Orientation on mathematical modeling in biology”	2011	1.5
MSc. level courses		
Course “Anatomy and Physiology”	2011	7.0
Statutory Courses		
Use of Laboratory Animals (article 9 authorisation)	2007	3.0
Professional Skills Support Courses		
Course “Techniques for Scientific Writing”	2005	1.0
Provimi Potential Managers Meeting (Amsterdam, 17-19 jan 2011)	2011	1.0
Provimi Potential Managers Meeting (Warsaw, 23-25 may 2011)	2011	1.0
Research Skills Training		
Preparing own PhD research proposal	2007	2.0
Visit to UCD Ireland for SF6 technique course	2007	1.0
Didactic Skills Training		
5 MSc. major students	2007-2011	10.0
2 BSc. students	2007-2011	2.0
Total		45.5

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